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Inclusion Bodies in Hakuri

By

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(Received for publication, August 1, 1959)

The disease under study has been known under a variety of names, commonly under the name of "pseudocholera infantum" since 1910. By the author (A. Sakamoto), however, Hakuri (=white diarrhea in Japanese) has been proposed as the term of this disease since 1956, because the term of pseudocholera was frequently or potentially confused with Asiatic cholera.

Hakuri is a seasonal, infantile diarrhea characterized by milky white stools, vomiting, and mild respiratory symptoms. Generally it runs a course of about one week followed by complete recovery. It occurs strictly during the winter months, and attacks only infants epidemically or sporadically. The present paper reports the existence of inclusion bodies, as yet unidentified, observed in a number of postmortem organs of Hakuri.

Methods and Material

On 7 cases of Hakuri autopsies, many stained impressions or smears prepared from organs were observed microscopically. All died between the 3rd and 19th day of illness. The specimens were stained by Giemsa's, Wright's, and Macchiavello's methods.

Microscopical findings[1)]

a) Intranuclear inclusions

They appeared stellate in shape and were surrounded by a clear halo (Fig. 1). The bodies stained basophilic, the same color as that of chromatin, with no dramatic changes in the nucleus (Cowdry Type B), and were located peripherally as a rule. Usually a single inclusion body is detected in a cell. The bodies were demonstrated in 3 out of the 7 cases studied.

b) Intracytoplasmic inclusions

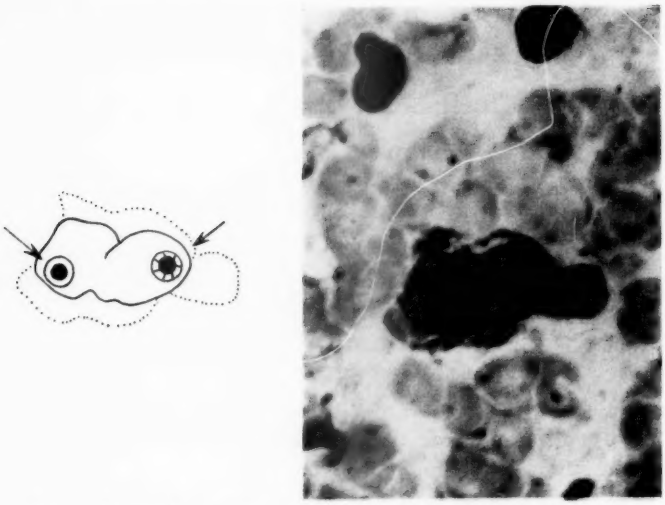


Fig. 1. Intranuclear bodies in a giant cell found in smear from the spleen. Giemsa's stain.

They were acidophilic round granules, about 200–300 mμ in size, and were seen multiply (Fig. 2) or singly, in pairs, or in groups. A clear hof may be observed around a particle. The granules may be found free outside the cells. Often these particles accumulate and form a spherical larger body (hereafter termed a globular body), usually 2–3 μ in diameter, and as large as an erythrocyte at the largest. Most of these globular bodies appear granular in structure and are present within a cell or freely. They may occasionally take the appearance of a nearly homogeneous stained body, within which many virus particles are presumably occluded. In particular, globular bodies were found numerously in the livers of 2 cases by Macchiavello's stain: there were seen many large vacuoles, in the center of which acidophilic granular material are seen in clusters. On the other hand, such clusters have not been detected in similar specimens stained by Giemsa's, or Wright's stain. It has not been established whether these clusters are related to the causative agent. The above mentioned inclusions were encountered in all 7 autopsy cases, specifically in mononuclear cells, reticuloendothelial cells, and epithelial cells (found in the bronchus, gall-bladder and intestine). In a number of organs studied the body was most frequently demonstrated in the mesenteric lymph nodes, the livers, the spleens, and less frequently the lungs, occasionally in the stomach, intestine, cervical lymph nodes, and in the bile found in the gall-bladder.

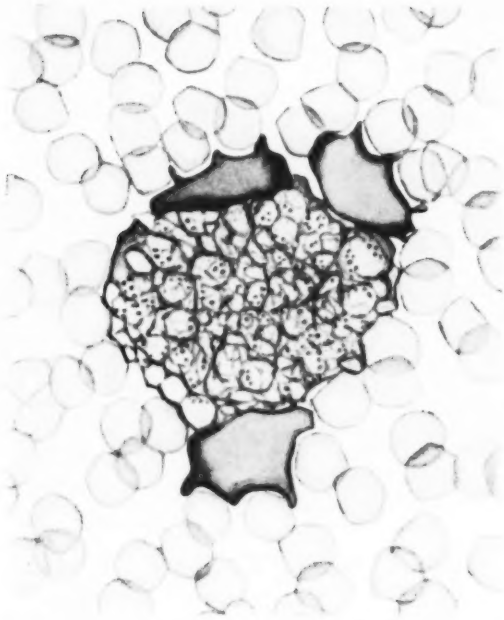


Fig. 2. Colored drawing of multiple cytoplasmic particles found in a vacuolated histiocyte. Lung smear stained by Giemsa's method.

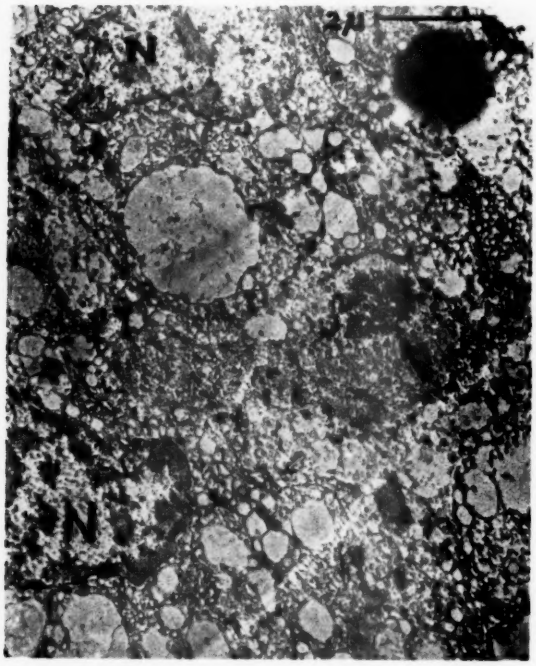
In general it is not easy to detect both inclusions; one hour's patience is necessary before they are located. It should also be noted that cytoplasmic inclusions or elementary body-like particles vary fairly widely in size. It is possible that the causative agent appears to develop the multiplication cycle. Concerning the staining methods Macchiavello's stain was the most preferable.

The inclusions under question must not be confused with thrombocytes particularly, also with granules of mastcells, eosinophilic or neutrophilic granules, and with secretion granules in the epithelium of the gastrointestinal tract.

Electron microscopic findings

With the autopsy materials an attempt was made to detect virus-like particles or globular bodies. The study is still in progress.

As is shown in Figs. 3 and 4, a globular body (2 μ in diameter) was demonstrated by electron microscopic examinations of thin sections of the liver of a patient (Hattori). The body was filled with virus-like particles (60–70 mμ), which appeared to show inner structures by further magnifi-



N = Nucleus

Fig. 3. Thin section of a patient's liver, showing "a globular body" (2 μ in diameter) in the cytoplasma. ×8,000

cation. The globular body probably belongs to homogenous stained bodies seen under the ordinary microscope.

Discussion

The etiology of Hakuri remains unknown. The above data, however, suggest strongly that the disease should be of viral origin. In addition, clinical data support this, and will be published in greater detail later. The cardinal pathologic picture of the disease consists of, (1) more or less medullary swelling of the mesenteric lymph nodes, (2) enteritis follicularis, (3) no enlargement of the spleen, (4) activities of both reticuloendothelial cells and lymphatic systems of (1) (2) (3), (5) no liver enlargement, showing frequently the picture of serous hepatitis and no evidence of bile stagnation, (6) evidence of interstitial pneumonia, (7) marked edema and hyperemia of the brain. On the basis of these changes in the organs it may be concluded that the disease is connected with an acute infectious disease entity involving the intestinal tract, particularly the upper pro-

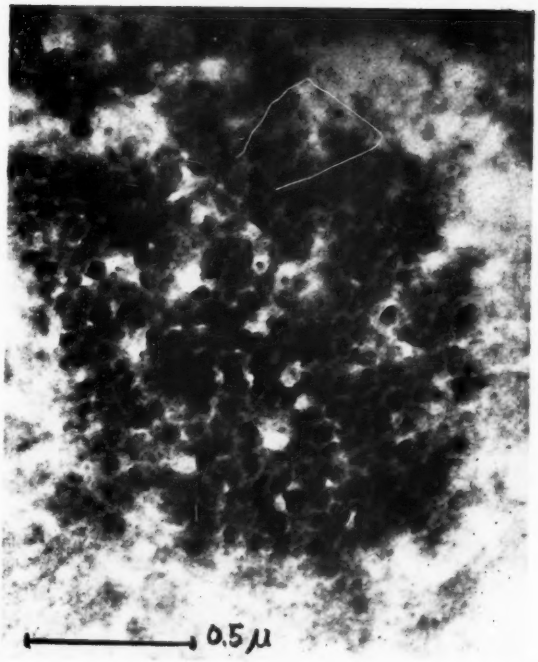


Fig. 4. High magnification of the globular body (↙) of Fig. 3, showing that several hundred virus-like particles (60–70 mμ) fill it. ×54,000

portion.

Hakuri is distributed throughout Japan, involving Korea, Taiwan, and Peiping, but has not been reported in Europe and America.

Differentiation can easily be made from other known inclusions (primary pneumonitis of Adams,[2] some types of adenoviruses,[3] cytomegalic inclusion disease, giant cell pneumonia[4]), by special attention to their preferable locations.

Summary

Based on 7 Hakuri autopsies, new(?), prominent intracytoplasmic (elementary body-like particles and globular bodies) and less prominent intranuclear inclusions were demonstrated in smears from organs by Macchiavello's, Giemsa's, and Wright's stains. With the electron microscope a globular body found in the liver of a patient was proved to be composed of many virus-like particles which occluded the body. Both inclusions were most frequently detected in the mesenteric lymph nodes, the liver, the spleen, the lung, and occasionally in other organs.

The authors wish to express their gratitude to Prof. K. Hukai for the electronmicroscopic examination.

This study was aided by a grant from the Ministry of Education.

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Sympathetic and Vagal Discharges in the Cardiac Nerve of the Toad

By

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(Received for publication, August 15, 1959)

For the understanding of the mechanism of the neural control of the heart, observations of efferent discharges in the cardiac nerve, both sympathetic and vagal, are indispensable. In 1936, Bronk, Ferguson, Margaria and Solandt[2] observed that efferent impulses were incessantly sent in the cardiac sympathetic nerves of the cat. This was taken as positive evidence for the view expressed by Reid Hunt[7] that the heart is under a continuous barrage of sympathetic impulses. Thereafter, sympathetic discharges in the cardiac nerve have been studied by several authors[1,3,4]. However, very few authors have treated them in relation to the cardiac reflex and no attempt has ever been made to record vagal impulses in the cardiac nerve.

The author found sympathetic tonic activity in the cardiac nerve of the toad and studied how it was influenced reflexively by electrical stimulation to the central end of various nerves. In addition, though seasonal changes were considerable, vagal impulses were also recorded in the cardiac nerve and could be related to the classical Goltz reflex. In the latter part of this paper some effects of the sympathetic and vagal impulses on the heart are also described.

Method

A toad weighing between 200 and 300 g. was anesthetized with urethane and fasten back down on a board. The heart was exposed by a median incision through the pectral girdle and the linea alba. After slitting open the pericardium, the heart was reversed as is shown in Fig. 1 so that the sinus and the caval veins might be exposed. The cardiac branch (arrow) of the vagosympathetic trunk enters the sinus venosus in a fine strand at the root of the pulmonary vein on each side. This nerve was cut as distally as possible and dissected free from the surrounding tissue in such a manner that it might be slung on a pair of silver-silver

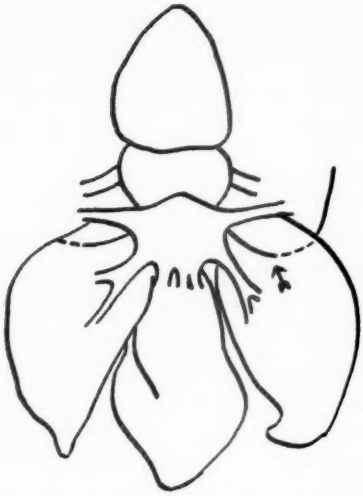

Fig. 1. Cardiac branch of vagosympathetic trunk (arrow). After slitting open the pericardium the heart was reversed to expose the sinus and caval veins.

chloride electrodes for recording action potentials. Precautions were taken to avoid damaging the sinus or adjacent large vessels. As this nerve penetrates also the pulmonary tissue in most instances as is shown in a dotted line in the figure, the lung had to be partially destroyed during dissection. Occasionally, such a cardiac branch was not found probably because of its arborization into finer strands on its way to the heart. However, in the majority of animals, a nerve thick enough to permit macroscopic dissection was found at least on one side.

Action potentials of the nerve and the heart were amplified by CR-coupled amplifiers and observed and recorded by either a cathode ray oscilloscope, an electromagnetic oscillograph or pen-recorder. Electrical stimulation of the nerve was performed with square pulses isolated from earth.

Results

Tonic discharges

In Fig. 2 the efferent discharges recorded from the cardiac nerve are presented. Without any stimulus, such an incessant spontaneous activity was always observable. In the toad most sympathetic fibers supplying the heart leave the spinal cord by the ventral roots of the 2nd and 3rd spinal nerves, pass the rami communicantes, change the neurons at the corresponding sympathetic ganglia and ascend in the sympathetic chain

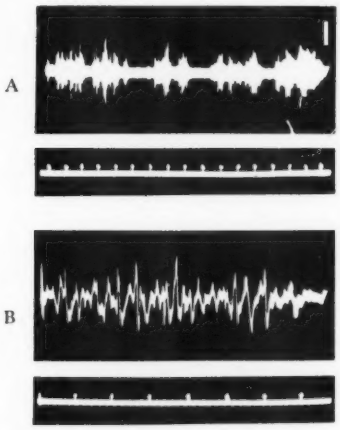

Fig. 2. Tonic discharge in cardiac nerve. Time mark: 1 cps. in A and 10 cps. in B. Voltage scale: 100μV.

to the jugular ganglion. There they join the vagal nerve and go to the heart in the vagosympathetic trunk. Therefore, the severance of the sympathetic chain just below the jugular ganglion can eliminate the sympathetic impulses to the heart. It was found that the spontaneous impulses observed in the cardiac nerve are mostly sympathetic in origin, because impulses were quite rare in the cardiac nerve after the aforementioned severance. Most of the remaining impulses were not vagal in origin but ascribable to the after-discharge from the cut end or to the spontaneous activity of the intercalated sympathetic ganglion cells, because a discharge of an almost similar amount was still observed after further severance of the vagal roots in the cranial cavity. Therefore, while the presence of the sympathetic tonic discharge in the cardiac nerve is clear, the vagal tonic discharge could not be demonstrated by this method. In the latter part of this paper, however, a certain evidence for the presence of the vagal tonic discharge in some animals is presented.

The pattern of the sympathetic discharge in the cardiac nerve was similar to that in the splanchnic nerve of this animal which was reported by the author previously[8]. The conduction velocity was also of the same order (0.4 m./sec. on the average at 18°C). In addition, a simultaneous recording of both cardiac and splanchnic discharges revealed that they not infrequently waxed and waned synchronously (Fig. 3). A similar synchronism was also observable between right and left cardiac nerves. And, similar to the splanchnic discharge, the cardiac discharge often grouped in phase with the heart beat.

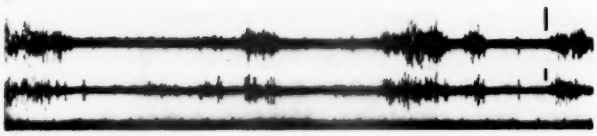

Fig. 3. From above downwards, discharge in the cardiac nerve, that in the splanchnic nerve and time mark (1 cps.). Voltage scale: each 100 μV. Note synchronism between the two discharges.

Reflexive changes of sympathetic discharge on nerve stimulation

On electrical stimulation of various nerves, the discharge in the splanchnic nerve of this animal was at first increased and then inhibited[8]. And, after the severance of the medulla oblongata at the level of the calamus scriptorius, stimulation evoked only the increase of discharge and the inhibition was no longer observable. This was also the case with the sympathetic discharge in the cardiac nerve. The records reproduced in Fig. 4 were obtained from the right cardiac nerve of a toad

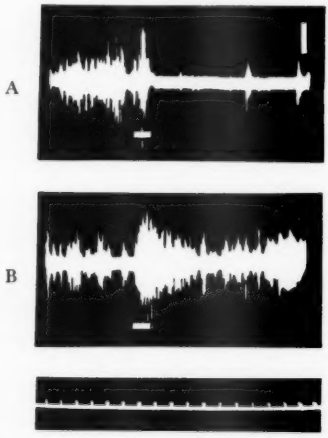

Fig. 4. Effects of electrical stimulation of splanchnic nerve on sympathetic discharge. A. The ipsilateral splanchnic nerve was stimulated at 50 pulses/sec. for the underlined period (1 sec.). The discharge was first increased and then inhibited for several seconds. B. The same stimulation after severance of the medulla oblongata. Inhibition was no longer observed. Time mark: 1 cps. Voltage scale: 100 μV.

on stimulation of the right splanchnic nerve at 50 pulses/sec. for 1 sec. By stimulation, a discharge greater than the spontaneous discharge in amplitude was evoked and, thereafter, the spontaneous discharge was inhibited for several seconds. Record B was taken using the same stimu-

lation after the removal of the medulla oblongata and other parts of the brain by a horizontal cut at the level of the calamus scriptorius. In contrast to A, stimulation evoked a marked increase of discharge only.

Though a small reflexive discharge was obtained by a single pulse stimulus, it was usually masked by the tonic activity. At a faster sweep speed as in Fig. 5, however, when a single pulse stimulus fell into a relatively silent phase of the spontaneous discharge, it was possible to observe

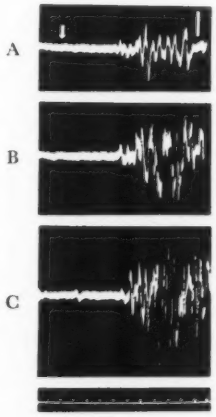

Fig. 5. Latency for evoked discharge. The splanchnic nerve was stimulated by a single pulse stimulus at the arrow in A, by four pulses at 20 msec. intervals in B and at 50 pulses/sec. for 1 sec. in C. Time mark: 20 cps. Voltage scale: 100 μV.

the response and to estimate its latency. In A of Fig. 5 the reflexive discharge made its appearance about 200 msec. after a single pulse stimulus given to the ipsilateral splanchnic nerve. Taking into consideration the relatively long efferent pathway (about 5 cm.) and the slow conduction velocity, it was found that a delay of 50–100 msec. occurred in the central nervous system. In B of Fig. 5, the splanchnic nerve was stimulated with 4 shocks at 20 msec. intervals and in C at 50 pulses/sec. for 1 sec. The amount of the discharge evoked increased with the increase of the number of the given pulses. However, the latency did not appear to change greatly.

In the above stated experiments stimulation was performed with square pulses of 5 V.–0.5 msec. Observation of the direct action potentials in the splanchnic nerve revealed that this stimulus was nearly supramaximal. At 2 V., only a group of nerve fibers with the fastest conduction velocity were stimulated. When the splanchnic nerve was stimulated at this

strength, some reflexive discharge could still be evoked in the cardiac nerve.

In the splanchnic nerve and the other sympathetic nerves communicating with the plexus ischiadicus, the efferent discharge was increased and then inhibited, regardless of whether the (contralateral) splanchnic nerve, the vagosympathetic trunk, the spinal nerve, or the trigeminal nerve was stimulated. And this effect was always bilateral: stimulation of any of the above stated nerves on either side could evoke similar responses in bilateral splanchnic nerves. This fact also holds in the case of the sympathetic discharge in the cardiac nerve. The reflexive change which was similar to that obtained on stimulation of the splanchnic nerve could be evoked by stimulation of the spinal nerve, the vagosympathetic trunk or the trigeminal nerve on either side.

Vagal discharge

The severance experiment revealed that the spontaneous as well as evoked discharge in the cardiac nerve was mostly sympathetic in origin. On stimulation of the splanchnic nerve, however, when observing at a relatively fast sweep speed, a reflexive discharge of a fast time course and of a small amplitude sometimes preceded the sympathetic discharge (Fig. 6 A). This was vagal in origin, because it was still observable after sever-

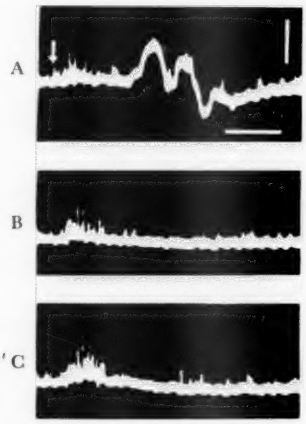

Fig. 6. Vagal discharge in the cardiac nerve. A. At the arrow a single pulse stimulus was given to the splanchnic nerve, which evoked vagal discharge of shorter latency and smaller amplitude compared to sympathetic discharge which followed. B. After severance of sympathetic chain just below the jugular ganglion. The stimulus was the same as in A. Only the vagal discharge was observed. C. Four pulses were given to the splanchnic nerve at 20 msec. intervals. Time scale: 100 msec., Voltage scale: 100 μV.

ance of the sympathetic chain just below the jugular ganglion (Fig. 6 B and C) and it disappeared after the intracranial vagal roots had been cut. Whereas the above stated sympathetic discharge could be observed in any season of the year, the seasonal change of the vagal discharge was considerable. By a series of observations from April of 1957 to March of 1959, it was found that the vagal discharge was elicitable most easily in November and in February. In other months of the year the discharge was extremely small if present at all. Furthermore, there was great diversity among animals, even in the above stated months, in the facility with which the vagal discharge could be evoked. It appeared that, in general, the discharge was more easily evoked in the male than in the female toad.

When the splanchnic nerve was stimulated on one side, the vagal discharge was observed almost to the similar extent on both sides. However, in contrast to the sympathetic discharge, the vagal discharge was not evoked by stimulation of other nerves such as the 6th spinal nerve and the sciatic nerve.

Effects of efferent impulses in the cardiac nerve on the heart

Severance of the bilateral cardiac nerves usually results in slowing of heart beat. Presumably this should be ascribed to elimination of the tonic accelerator impulses in the sympathetic nerves. Occasionally, however, the severance increases heart rate. It may be said that, in such animals, the vagal tonus has been enhanced. Bearing in mind the difference of the routes of vagal and sympathetic nerve fibers supplying the heart, if each of them were severed separately, it is possible to understand why this diversity arises. In the animal from which the records in Fig. 7 were

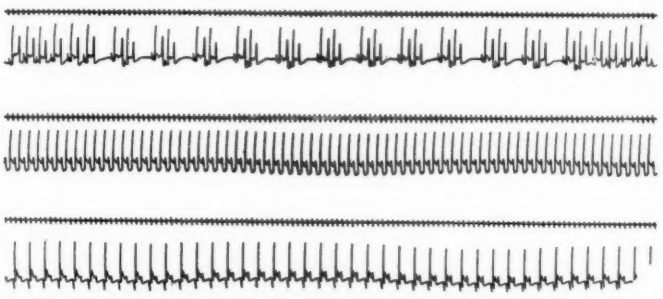

Fig. 7. Electrocardiogram recorded from a toad. A. Prior to any intervention to the cardiac nerve. B. After severance of the bilateral vagal roots in the cranial cavity. C. After further severance of the bilateral cardiac nerves. Time mark: 1 cps.

taken, the arrhythmia demonstrated in A was present prior to any intervention to the cardiac nerve. After elimination of vagal impulses by severance

of the bilateral vagal roots in the cranial cavity, the arrhythmia disappeared and the heart rate increased (B). After that the cardiac nerves were cut on both sides so that even sympathetic impulses failed to reach the heart. As is shown in Fig. 7 C, this procedure brought about a slowing of heart beat.

An electrocardiogram recording made on stimulation of the central end of the splanchnic nerve frequently showed slowing or standstill of the heart in the season when the above stated vagal discharge was observable. In Fig. 8 A, the right splanchnic nerve was stimulated at a

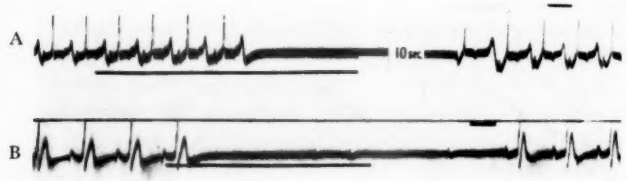

Fig. 8. Effects of vagal discharge on heart. A. For the underlined period a 50 pulses/sec. stimulus was applied to the splanchnic nerve, which evoked a reflex inhibition of the heart. The record is omitted for 10 sec. when the heart was in standstill. B. The same experiment in another toad, showing the appearance of AV block during the inhibition of the heart. Time scale: 1 sec. both in A and B.

frequency of 50 pulses/sec. for the underlined period. During stimulation the heart stopped and resumed beating about 12 seconds after cessation of stimulation. (A portion of the record was omitted for 10 seconds when the heart was in standstill). Usually, stimulation for briefer intervals was effective and, in some animals, a single pulse stimulus to the central cut end of the splanchnic nerve induced a slowing of heart beat. In B, during and after stimulation, only the P wave made its appearance without the following ventricular complex, indicating the presence of AV block.

In the animals in which this reflex inhibition of the heart evoked, it could be repeated at several minute intervals. Though this reflex was still obtainable after severance of the vagal roots only on one side, bilateral severance abolished it. Therefore, the efferent arc of this reflex can be attributed to the vagal outflow in the vagosympathetic trunk. As stated before, on stimulation of the splanchnic nerve, the response observed in the cardiac nerve was mostly sympathetic in origin and the vagal impulses were rare (Fig. 6). However, we have to conclude that these vagal impulses, though small in number, could evoke the marked inhibitory effect on the heart.

To examine the effect of the sympathetic reflexive impulses on the heart, bilateral vagotomy was necessary because the effect of vagal im-

pulses was as pronounced as stated above. After vagotomy splanchnic stimulation had almost no effect on the heart rate. On the assumption that the inhibition of the sympathetic discharge which followed the evoked discharge might cancel the effect of the latter, the brain higher than the medulla oblongata was removed so that stimulation might evoke only an increase of sympathetic impulses as is shown in Fig. 4 B. Even after this procedure, the central splanchnic stimulation induced no appreciable change in heart rate. However, when the condition of experimental animals was worsened after a long-lasting experiment and the activity of the heart was deteriorating, improvement of the activity was occasionally observed on stimulation of the splanchnic nerve for a relatively long period. In Fig. 9 A, AV conduction reappeared on stimulation of the splanchnic

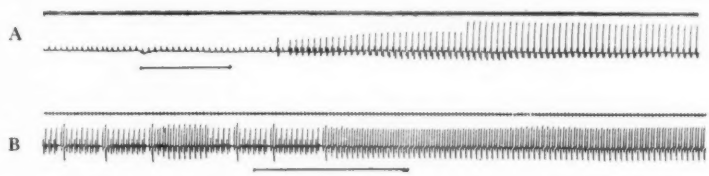

Fig. 9. Effects of sympathetic discharge on heart. The animals were vagotomized prior to the experiment. Both in A and B the central splanchnic stimulation at 50 pulses/sec. was performed for the underlined period. AV conduction was improved. Time mark: 1 cps.

nerve in a case in which AV block had developed. Record B was taken from another toad in which an incomplete AV block was improved by stimulation. In either case AV block reappeared as before several minutes after the cessation of stimulation and the above stated effect could be reproduced several times by repeated stimulation. This effect was not confined to the splanchnic nerve only and a similar effect was also obtained by stimulation of the 6th spinal nerve.

Discussion

The sympathetic discharge in the cardiac nerve dealt with in this study bore a close resemblance to that in the splanchnic nerve in the discharge patterns, in the conduction velocities and in the responses to electrical stimulation of various nerves. Furthermore, as is shown in Fig. 3, the efferent discharges in these two nerves often waxed and waned in parallel and, on stimulation of an appropriate nerve, they responded by a synchronous increase of discharges followed by inhibition. The diffuse and synchronous nature of the activity of the sympathetic nervous system is usually assumed on the basis of the parallel change of the activity of

various effectors. The author supposes that the result demonstrated in Fig. 3 furnishes direct evidence for this view. However, this synchronism was not a constant findings and it is still not clear under what condition this synchronism was induced. The mechanism of the synchronism between the bilateral cardiac nerves is also still obscure. On the basis of the observations so far accumulated, however, at least the following inference might be possible. In the previous study[8] the author suggested a hypothesis in which he attributed an automaticity to the sympathetic cell group at the segmental level and assumed a structure in the upper part of the spinal cord upon which afferent impulses converge to induce an increase of the splanchnic discharge. Another structure was assumed in the medulla oblongata which is concerned with the inhibition of the splanchnic discharge. The results obtained in the present study indicate that such structures also control the cardiac sympathetic discharge in parallel with the splanchnic discharge and may be referred to as sympathetic cardiovascular centers. It may be assumed that the synchronism between the cardiac and splanchnic discharges or between the bilateral cardiac discharges is maintained by the activity of these centers and that asynchronism is attributed to a lowering of their activity which results in appearance of automaticity of each segment on each side.

It may be said that the afferent pathway for the sympathetic response described in this paper is quite non-specific, because stimulation of most nerves hitherto tested was found to evoke almost similar effects. A certain kind of afferent fibers of each segment seems to be connected to the above stated sympathetic centers through the intermediary of an intraspinal neuron or a series of such neurons. On the other hand, it appeared that the afferent pathway of the vagal response was quite specific and confined to the splanchnic nerve. Though the vagal discharge was weaker than the sympathetic discharge when observed in the cardiac nerve, the former produced much more marked effects on the heart than the latter. The vagal impulses, though scanty in number in the extracardiac nerve, may be mediated to many postganglionic fibers in the intracardiac ganglions[6] to effect a marked inhibition.

From Goltz's detailed observations[5], it is almost certain that the afferent pathway of his reflex is in the splanchnic nerve and the efferent pathway in the vagal nerve. The author attempted to analyze this reflex by the electrogram of the cardiac nerve, standardizing the afferent impulses by electrical stimulation. However, seasonal changes of toad's response on the one hand and the diversity among animals in the susceptibility to reflex cardiac inhibition on the other made this a difficult task. Goltz failed to obtain reflex inhibition of the heart on electrical stimulation of the splanchnic nerve. According to the author's impression, to evoke the

cardiac inhibition, the electrical stimulation of splanchnic nerve was far less effective than the conventional tapping of the abdomen. Presumably, tapping stimulates only the receptors for the reflex most effectively, while electrical stimulation excites in addition unnecessary fibers which might have antagonistic effects. Or, alternatively, unnatural pattern, both temporally and spatially, of the splanchnic impulses elicited artificially by electrical stimulation may not be appropriate for induction of this reflex.

Summary

1. Efferent impulses of the cardiac nerve were studied in the toad, in order to relate them to the cardiac reflex in this animal.

2. Tonic discharges observed in this nerve were mostly sympathetic in origin and were similar to those in the splanchnic nerve in discharge patterns, conduction velocities and responses to electrical stimulation applied to the central end of various nerves. Not infrequently, the discharges in these two nerves waxed and waned synchronously.

3. From the cardiac nerve of some animals, there was also recorded a vagal discharge which effected slowing or standstill of the heart. Such a vagal discharge, however, was not a constant finding, its occurrence being dependent upon the season or upon the individual animals.

4. Severance experiments disclosed that at least one of the functions of the sympathetic tonic discharge was accelerating of the heart. Vagal tonic activity was also demonstrable in some animals.

The author wishes to express his great appreciation for the valuable comments and criticisms on the manuscript by Professor K. Matsuda.

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Nonspecific Resistance and the Reticuloendothelial System*

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The term ' nonspecific resistance ' describes all those defense mechanisms of the host which operate independently of the nature of the invading antigen, a characteristic which sets them clearly apart from specific means of defense as exemplified by antibodies. Nonspecific resistance had undoubtedly served as an essential concept of clinical medicine for a long time before its existence was experimentally demonstrated by Louis Pasteur in his famous presentation before the Academy of Medicine in Paris on March 19, 1878, just a little more than eighty years ago. He showed that chicken which are resistant to anthrax become susceptible to the disease if their feet are kept in a waterbath whose temperature lies below the body temperature of the chick.[1] The brilliant results, however, which were obtained in the field of specific immunology kept the general interest focused upon the phenomena of antigen-antibody interaction in spite of Metchnikoff's description of phagocytosis four years later, and it was not really until the first decade of this century that a period of extensive research in nonspecific defense mechanisms began.

Any attempt at a dependable classification of nonspecific resistance is beset with difficulties due to the lack of a precise definition of the subject, but it seems permissible and sufficient for all practical purposes to differentiate the following categories[2]:

Firstly: Resistance factors which are not amenable to outside influences—as age and genetic characteristics,

Secondly: Resistance factors which are amenable to outside influences. In this category, two groups may be recognized:

a) those factors which appear as a reaction against an invading microorganism as local inflammatory reaction, phagocytosis, activation of the reticuloendothelial system, production of bactericidal agents in serum, cells and tissues, and, perhaps, the Shwartzman phenomenon.

b) those mechanisms whose presence can only indirectly be demonstrated. Paradoxically enough, they must be defined—as long as their

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true nature is not understood—by the very factors which modify them such as nutrition, temperature of the environment, radiation, weather etc. It seems likely that they are connected with the metabolism of the host.

How important each of these factors is in the establishment of an equilibrium between host and parasite is quite difficult to decide. For some of them it still remains to be proved that they have any connection with the phenomenon of resistance at all. This is not surprising if one takes the obstacles into account which the investigator has to overcome who wishes to approach this field experimentally. There is hardly ever a single factor operating so that the presence of other, perhaps equally important factors cannot be excluded.

The present paper deals with only one aspect of host defense, and although this might probably be the most extensively examined, it must be viewed as one among many. After Metchnikoff's description of phagocytosis in 1882 the idea quickly gained acceptance that an invading microorganism would be ingested by mobile tissue and blood cells and, as a rule, be destroyed.[3)4)] Wyssokowitsch, however, who was the first investigator to observe bacteria inside of Kupffer cells of the liver and endothelial cells of the splenic sinuses in the rabbit, postulated that fixed cells were much more important in taking up particulate matter which had gained access to the bloodstream.[5)] At the same time, the introduction of vital staining opened a new avenue of approach. Beginning with Heidenhain in 1878, a number of authors such as Ribbert, Schlecht, Pari, Fujinami, Suzuki, Masuka and Oka demonstrated that colloidal dyes as for instance indigocarmine, lithiumcarmine, trypan blue and red, were stored in a variety of cells. These included endothelial cells of the blood vessels, the convoluted tubules of the kidneys, fibroblasts, parenchymal cells of the liver etc., depending upon the frequency of injections and the type of dye used.[6)] The most outstanding results were achieved by Goldman who injected pyrrhol- and isaminblue into the peritoneal cavity of guinea pigs.[7)] A great step forward was made in 1914 when Kiyono classified for the first time the cells which were capable of storage and brought order into the confusing variety of observations. Based upon his findings with lithium carmine in the rabbit he concluded that in the normal animal the Kupffer cells of the liver, endothelial cells of the splenic and lymphatic sinuses, bone marrow, anterior pituitary gland and adrenal vessels represented the foremost sites of storage. He separated them clearly from the histiocytic elements in the spleen and loose connective tissue whose capacity to store was considerably less.[6)]

In summing up the results of Kiyono's investigations, Aschoff and Landau coined the term 'reticuloendothelial system' which soon was found of practical importance when Anitschkoff demonstrated the role of

the Kupffer cell in the metabolism of cholesterol.[8] Only a few years later, Rous and Robertson observed the destruction of aging red blood cells in the sinuses of the spleen,[9] and numerous workers began to concentrate their interest upon the function of the reticuloendothelial system in experimental or clinical infection.[10] It quickly became known that injection of carbon in the form of India ink, vital dyes, certain iron salts and other substances could change the course of an experimental infection for the worse. It was generally assumed that these materials were able to block the reticuloendothelial cells and thus to prevent them from clearing the blood of circulating bacteria.[11–18]

It is difficult to conceive how this hypothesis could find so widespread an acceptance since it was well-known from the beginning that a) this result could only be achieved for a very limited time with unusually high doses of " blocking material " and never in all instances, b) many reticuloendothelial cells remained free of the " blocking substance " and c) that reticuloendothelial cells can contain both " blocking substance " and bacteria. In 1934, v. Jancso suggested therefore the use of electrolytically dispersed copper for elective destruction of reticuloendothelial cells.[19] Although his method appears until to-day the only one which works dependably it was never widely employed. At about the same time the radioactive compound thorium dioxide (thorotrast) was found to be stored in the reticuloendothelial system and became rapidly popular among research workers[20][21]. It remained so until now although its mode of action still awaits clarification.

Even if the behaviour of the reticuloendothelial system under the influence of ' blocking ' agents must be considered an unsolved problem it nevertheless serves to illuminate the important role which these cells play in the defense of the host. This role was found not only in the realm of nonspecific, but also in that of specific resistance when McMaster and Kidd in 1937 and Florence Sabin in 1939 proved that reticuloendothelial cells are able to produce antibodies both in vivo and in vitro[22][23]. According to Houghton who observed antibody production in impure explants of human spleen cells it seems likely that this mechanism operates also in man[24].

While more and more insight into the functions of the RES was gained, the debate on the purely morphological aspects of this system went on for years without yielding conclusive results. The original descriptions by Wagner[25] and v. Kupffer[26] had been limited only to the phagocytizing cells of the liver. Kiyono, in his treatise ' Über die vitale Karminspeicherung ', differentiated wandering from sessile RE cells. The problem appeared to be solved already when he postulated that histiocytes, lymphocytes and monocytes could appear in the circulation and be grouped

together as ' histiocytic elements ', while Kupffer cells, endothelial cells of the splenic sinuses, bone marrow, adrenal cortex and the pituitary gland belonged to the reticuloendothelial system proper[6)27)]. This seemingly well-based and clearcut classification was challenged, however, when v. Möllendorf developed his theory of the fibrocyte as the stem cell for the formed elements of the blood[28)], which denied the existence of true histiocytes. It was not until the early thirties that Kiyono's, and, in a certain sense also Maximow's, theories were confirmed by tissue culture studies[29)30)]. To sum up the results of these extensive and laborious investigations it may suffice to say that histiocyte, monocyte and lymphocyte are only different, transient forms of differentiation, derived from the primitive reticular cell. It is well-established that the histiocyte develops most frequently out of a lymphocyte and can be transformed into a fibroblast. It is also likely that the reverse process, the transformation of fibroblasts or even fibrocytes into histiocytes, can take place[30)]. The ordinary endothelial cell, on the other hand, bears no relationship whatsoever to the histiocyte, although it is capable of storing vital dye particles after repeated injection or of taking up carbon after topical application of histamine[31)32)]. What position must be assigned to the Kupffer cells and the endothelial cells of the spleen in this general concept? Clearly, these are the most important reticuloendothelial cells: they show avid phagocytosis to a degree which is never attained by histiocytes, but form anatomically a part of the endothelium, regardless of whether one considers them lying inside[33)] or outside[34)] the capillary lumen or believes them to be accumulations of endothelial plasma only[35)]. De Haan and Hoekstra demonstrated in 1928 that monocytes which had engulfed trypan blue and subsequently been injected intravenously into guinea pigs, rats or rabbits, became trapped in the liver capillaries and occupied those sites which are usually taken by the Kuppffer cells[36)]. These observations were supported by the investigations of Hamazaki and Watanabe who administered lithium carmine to rabbits and, more recently, by Wachter who used India ink in guinea pigs. Their results militate against the view that the Kupffer cells are sessile and strongly suggest that they can be derived from circulating histiocytes or monocytes[37)38)]. It needs, however, to be emphasized that this hypothesis has not remained undisputed and that there are also investigators who consider the Kupffer cell as truly endothelial.

In this connection it seems surprising that the endothelial cells of the splenic sinuses have only occasionally received attention[39)], and even less so the other cells included under the name of the reticuloendothelial system. There is no doubt that the different elements of the RES, although they possess the common function of phagocytosis, display wide quantitative differences with regard to the amount of material which they

ingest. Under the influence of infection, for instance, the hepatic RE cells of the mouse increase their phagocytic capacity to a much greater extent than those of the spleen which at times is found almost entirely devoid of storing cells[40]. This phenomenon might explain why extirpation of the spleen in mice and guinea pigs failed to exert a negative influence upon the course of an experimental Bang-infection[41].

The kinetics of phagocytosis by Kupffer cells are partly known. Earlier investigators reported upon the fact that they ingest exclusively acid, electronegative dyes and that degree and rapidity of ingestion depend upon the state of dispersion of the dye. V. Jancso demonstrated in rats that phagocytosis by Kupffer cells takes place in two steps: particulate matter or dye is adsorbed to the cell surface first and consequently phagocytized. Tlis second step takes place only at normal body temperature[42][43].

Phagocytosis by Kupffer cells is not qualitatively different from the phagocytic process seen in neutrophils and is, in certain bacterial species, effectively aided by opsonins[16][44]. Rowley, Howard and Wardlaw showed that rat blood contains at least two opsonic factors which increase the ingestion of bacterial in the perfused ratliver. It is conceivable that it was the absence of such factors which prevented active phagocytosis by those Kupffer cells which had been isolated from dogs and cultured by Rous and Beard in 1932[45]. Recently more and more evidence has been accumulated which points to an almost specific effect of opsonic factors. Sheperd demonstrated that tubercle bacilli were taken up by HeLa cells only when they were suspended in horse serum[46].

Granulopectic activity of the reticuloendothelial system can be measured in various ways, for instance by the injection of azo-dyes, radioactive tracer substances or India ink with subsequent determination of the rate of clearance. The first attempts to measure RES activity quantitatively were made in 1925 by Saxl and Donath who used finely dispersed oil and Adler and Reimann who employed the azo-dye Congo Red. The value of these methods, however, was questioned[47] and they are at present only used by few investigators[48]. A dependable, semiquantitative test-method was described in 1947 by v. Jancso who used radioactive phosphate in mice[49] and two years later by Gabrieli who applied v. Jancso's method in rabbits[50]. The first extensive study of the kinetics of the reticuloendothelial system, however, were published in 1950 by Halpern, Biozzi, Benacerraf, Mouton and Stiffel who measured the disappearance rate of specially prepared India ink[51][52][53]. These authors established that the clearance of all particulate matter from the circulation is governed by two equations:

I. $C_t = C_o \times 10^{-kt}$ and

II. $k \times$ dose injected = constant,

where C_t represents the concentration of particulate matter in the circulation at a given time t after injection, while C_o signifies the concentration of the material at the time of injection (time O). k is the so called phagocytic index which will be explained below.

These equations are only valied if the size of the injected particles is kept within certain limits. Particles which measure below 80–90 Angström units are only poorly or not at all phagocytized, remain for a long time in the circulation and are in the end excreted via the kidneys. If a test substance is employed which contains particles of this type, as for instance many radioactive tracers do, it is necessary to deduct their respective concentration from the overall amount of injected material in order to arrive at the same results which are obtained with a uniform suspension of larger particles[54]. If, on the other hand, the size of the injected particles lies considerably above 1000 Angström units, pulmonary embolization can occur.

Text fig. 1 illustrates how equation I has been derived. If a certain amount of a stable suspension of India ink is injected intravenously into, for instance, a rat, one finds that the blood concentration of the injected material decreases in an exponential fashion. The curve becomes consequently a straight line if the logarithm of the concentration rather than the actual concentration is plotted against time. The slope which this line forms with the ordinate is expressed by the equation

$$k = \frac{\log C_o - \log C_t}{t} \text{ or } \log C_o - \log C_t = kt$$

This is the mathematical expression for the above mentioned phagocytic index k and indicates the rate at which a given particulate material is cleared from the circulation.

If this equation is converted into its numerical form equation I is obtained:

$$\begin{aligned}
\log C_o - \log C_t &= kt \times 1 \\
&= kt \times \log 10 \\
-\log C_t &= kt \times \log 10 - \log C_o \\
(-\log C_t &= kt \times \log 10 - \log C_o) \times -1 \\
\log C_t &= -kt \times \log 10 + \log C_o \\
C_t &= C_o \times 10^{-kt}
\end{aligned}$$

The phagocytic index k is not an invariable constant but changes with the dose employed which can likewise be recognized from text fig. 1. In addition, k depends upon the animal species which is used for testing as demonstrated by text fig. 2. The greatest relative decrease of k with respect to an increase in dose injected is observed in mice and rats. These animals

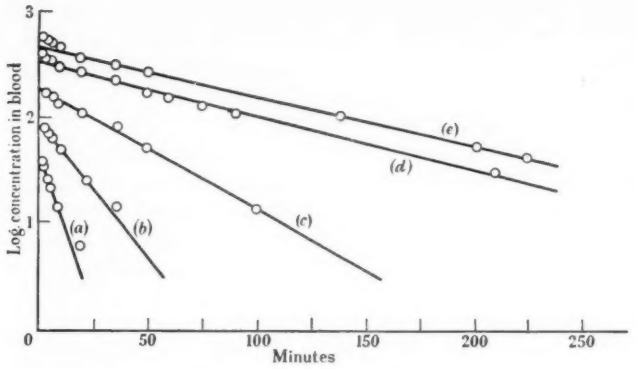

Fig. 1. Clearances of carbon from the rat after single doses. Concentrations of carbon expressed as their logarithms. a) 4 mg./100 g. body wt. b) 8 mg./100 g. body wt. c) 16 mg. per 100 g. body wt. d) 32 mg./100 g. body wt. e) 48 gm./100 g. body wt. From : Biozzi *et al.*, Brit. J. Exp. Path. 1953, **34**, 441.

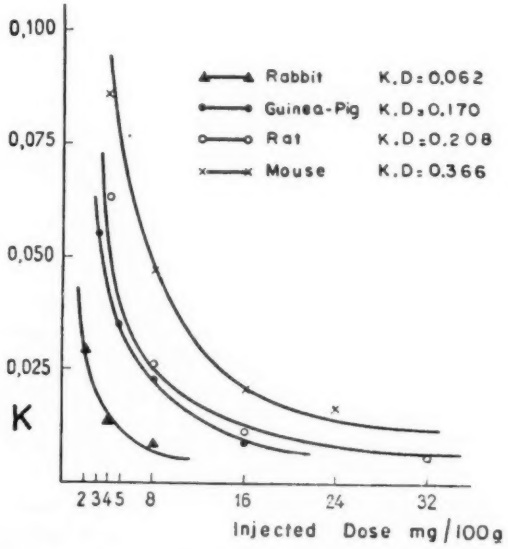

Fig. 2. Variations of the phagocytic index k with respect to the dose of carbon injected in various animal species. From : Benacerraf *et al.* in : Physiopathology of the RES, Blackwell Scientific Publishers, Oxford, 1957.

are therefore well-suited for experimentation with the reticuloendothelial system. Text fig. 2 permits at the same time to verify the validity of equation II, namely that the product of injected dose and phagocytic index k is constant for a given animal species. The values of this product are given in the upper right hand corner of text fig. 2.

The equations I and II permit us further to calculate the speed with which the injected test material, for instance carbon, is taken up by the RES. If the rat is used as experimental animal, the dose of injected carbon can be expressed as initial blood concentration × blood volume (rat 8 cc.). If this expression is inserted into equation II, one obtains

$$k \times C_o \times 8 = 0.208 \text{ or}$$
$$k \times C_o = \frac{0.208}{8} = 0.026.$$

Then, substituting k in equation I by this expression one finds

$$C_t = C_o \times 10^{-\frac{0.026 \times t}{C_o}}$$

In order to calculate the speed of phagocytosis this modified form of equation I must be derived with respect to t, i.e. for a change in concentration during the given time interval t

$$-\frac{dc}{dt} = k \times C_t \text{ or, substituting k,}$$
$$-\frac{dc}{dt} = 0.026 \times \frac{C_t}{C_o}$$

Changing the natural logarithm for the decimal logarithm, the equation is multiplied by 2.3 and one obtains

$$-\frac{dc}{dt} = 0.06 \times \frac{C_t}{C_o}$$

This expression needs now only to be modified with regard to the saturating effect of the carbon particles already ingested. If these phagocytized particles are called P, the concentration of carbon still present in the blood stream at the time t is $C_t = C_o - P$. Thus equation III is found

$$-\frac{dc}{dt} = 0.06 \times (1 - \frac{P}{C_o})$$

which is the general equation describing the phagocytosis of circulating particles except for very small doses which are cleared from the circulation in one passage through the liver. These very small doses have been indeed employed by Benacerraf et al. to measure liver blood flow in the rat[55].

Equation III is only valid up to a certain amount of carbon or par-

ticulate matter but within these limits it is permissible to state that the speed of clearance decreases with the amount of carbon injected and phagocytized. This is a relative, not an absolute phenomenon, because the rate of decrease diminishes with increasing dose of injected carbon. Needless to say, equations I, II and III possess validity only as long as the liver blood flow remains unimpaired. Any decrease in liver circulation is followed by a significant fall in phagocytic activity[55].

The liver occupies the most important place amongst the organs of the reticuloendothelial system. Only when concentration of particulate matter in the Kupffer cells has gone beyond a certain limit, storage in the spleen begins. Thymus, bone marrow, lymphnodes, adrenal glands and the pituitary gland play quantitatively only a negligible role compared with the first two organs. Therefore, the granulopectic activity of an experimental animal will vary for a given dose with the body weight in relation to the weights of liver and spleen as expressed by the quotient

$$\frac{\text{Body weight}}{\text{Liver}+\text{Spleen wts.}}$$

Text fig. 3 illustrates that this expression stands roughly in a third order relationship to the clearance factor k. Every point on this curve can thus be determined by the equation

$$\text{alpha} = \frac{\text{Body weight}}{\text{Liver}+\text{spleen wts.}} \times \sqrt[3]{k}.$$

The value ' alpha ', termed " corrected phagocytic index " has the advantage of showing a smaller standard deviation than the phagocytic index k which usually exhibits considerable variation. Whenever in the following discussion the term " RES activity " is used it means ability to clear intravenously injected particulate matter—here carbon—from the blood stream as measured by the equations I, II and III described above.

It is a well-established fact that dead bacillary matter in the form of vaccine or endotoxin endows the experimental animal with a considerable amount of protection against a subsequent infection with immunologically unrelated bacteria regardless of whether they are grampositive, gramnegative or—with limitations—acid-fast[56][57]. Pretreatment with pertussis vaccine conveys to the mouse effective protection against Kl. pneumoniae and virulent staphylococci. A lesser but still impressive degree of protection is obtained against Myco. tuberculosis.

This phenomenon is entirely nonspecific since it can be observed also after pretreatment with killed tubercle bacilli, extracts derived from them or after preinjection of a highly purified Salmonella endotoxin. Invariably, the protective effect becomes manifest before the appearance of

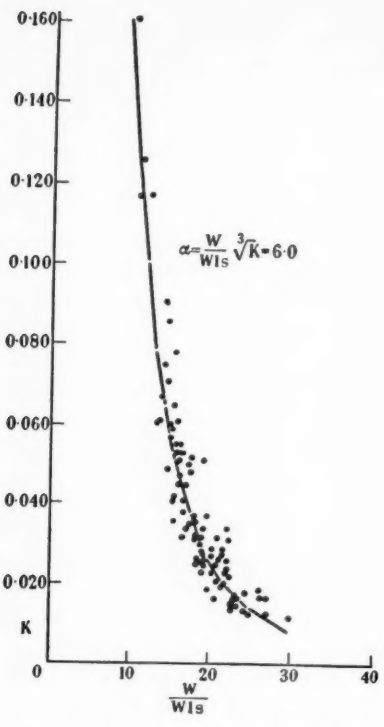

Fig. 3. Relationship between the phagocytic index k and the relative weight of the liver and spleen (body wt. divided by liver+spleen wt.) From: Benacerraf *et al.* in: Physiopathology of the RES. Blackwell Scientific Publishers, Oxford, 1957.

measurable antibodies.

The doses which convey protection to the mouse lie at 1/10 of the LD50, i.e. they are relatively high. They produce fever, leucopenia followed by leucocytosis, slowing down of blood clot formation, stimulate the adrenals and raise the granulopectic activity of the RES[58]. Text fig. 4 compares the effect of i.p. administered S. abortus equi endotoxin with killed tubercle bacilli (BCG). Both substances are able to raise RES activity considerably but the right hand side of text fig. 4 demonstrates that infection—in this case with Myco. fortuitum—provides a much more effective stimulus. It is interesting to note that young animals produce a much more marked response than older ones.

Intraperitoneal injection of a protective dose of endotoxin followed at

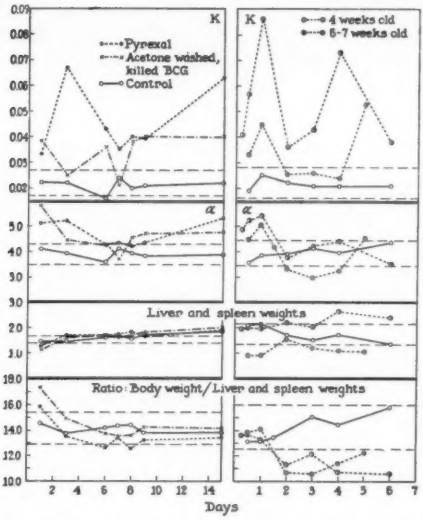

Fig. 4. Comparative effect of treatment with non-living bacillary materials and of infection with Myco. fortuitum on clearance rate of carbon particles. Each points corresponds to the arithmetic means of five animals, the broken line indicates the standard deviation for the untreated controls.

Left: S. abortus equi endotoxin, 20 micrograms, administered intraperitoneally in a final volume of 0.4 ml. of dist. water. Acetone-washed, killed BCG 200 micrograms, administered intraperitoneally in a final volume of 0.4 ml. of dist. water.

Right: Infective dose: 0.05 ml. of a 6 day old tween-albumin culture of Myco. fortuitum, strain Penso, injected intravenously in a final volume of 0.2 ml. of dist. water.

From: Boehme & Dubos, J. Exp. Med., 1958, **107**.

various time intervals by a challenge with Myco. fortuitum produces a rise in activity which surpasses the effect of each factor alone as seen in text fig. 5. Preinjection of endotoxin apparently endows the reticuloendothelial cells with an increased ability to respond to the subsequent infection and it has been shown that this increased responsiveness lasts for approximatively four to six weeks. Similar results can also be obtained with killed BCG bacilli as mentioned above.

Can one conclude from these findings that the increased resistance conveyed to the mouse by preinjection of endotoxin manifests itself exclusively through the increased granulopectic activity of the RES? No clearcut answer is possible since endotoxin produces a great many different effects and each of them may influence host resistance in various ways. It appears therefore necessary to exclude endotoxin from the experimental

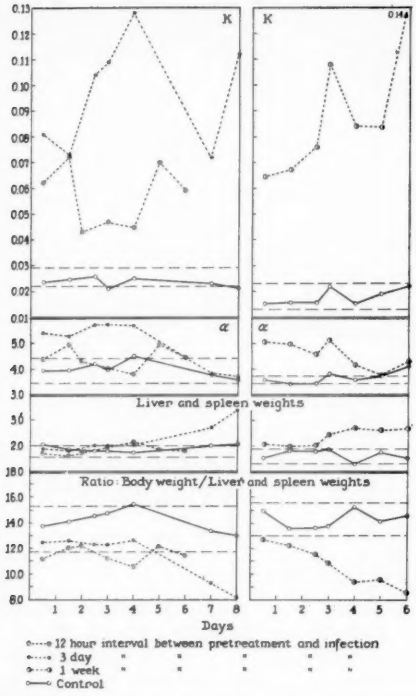

Fig. 5. Short term effect of infection on clearance rate of carbon particles in mice previously treated with S. abortus equi endotoxin. Each point corresponds to the arithmetic means of five animals. The broken line indicates the standard deviation of the untreated controls.

Left: One group of animals received 20 micrograms of S. abortus equi endotoxin intraperitoneally in a final volume of 0.4 ml. of dist. water and was challenged 12 hours later. The other group received an identical dose of salmonella endotoxin, but was challenged 3 days later.

Right: Treated intraperitoneally with 20 micrograms of S. abortus equi endotoxin in a final volume of 0.4 ml. of dist. water one week prior to challenge.

All three groups challenged with 0.05 ml. of a 6 day old tween-albumin culture of Myco. fortuitum—strain Penso—in a final volume of 0.2 ml. of dist. water.

From: Boehme & Dubos, J. Exp. Med., 1958, **107**.

model by using genetically resistant or susceptible mice whose status of resistance is determined by inbreading through successive generations.

Animals of this type are for instance the highly inbred BRVR and BSVS mouse strains from the Rockefeller Institute which are resistant resp. susceptible to an infection with S. typhi murium as clearly demonstrated

by text fig. 6. These two mouse strains exhibited definite differences with respect to the granulopectic activity of their reticuloendothelial systems which are recorded in text fig. 7. Contrary to expectations, the resistant animals (BRVR) did not show any rise in their RES activity until the eighth day after infection, while the susceptible (BSVS) mice responded almost immediately but were all dead on the eigth day. This result might be

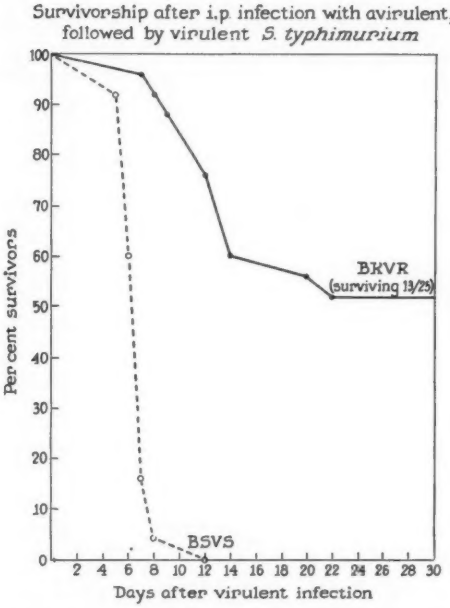

Fig. 6. Infected i.p. with 1000 viable cells of avirulent S. typhi murium, strain RIA, followed two days later by 100000 viable cells of the virulent strain SR-11.

From: Böhme, Schneider & Lee, J. Exp. Med., 1959, **110**.

explained by the assumption that in the resistant (BRVR) animals bacterial multiplication was delayed by factors other than the reticuloendothelial system and was therefore strong enough to stimulate the RES but not to tax it beyond its capacity. In the susceptible (BSVS) mice, however, bacterial multiplication went unchecked and was able to overwhelm cellular resistance as represented by the RES very quickly.

These findings certainly militate against the view that the RES occupies the most important position among the factors of nonspecific resistance. Very little is known about the way in which RE cells handle ingested bacteria. Biozzi showed that Kupffer cells break down a denatured

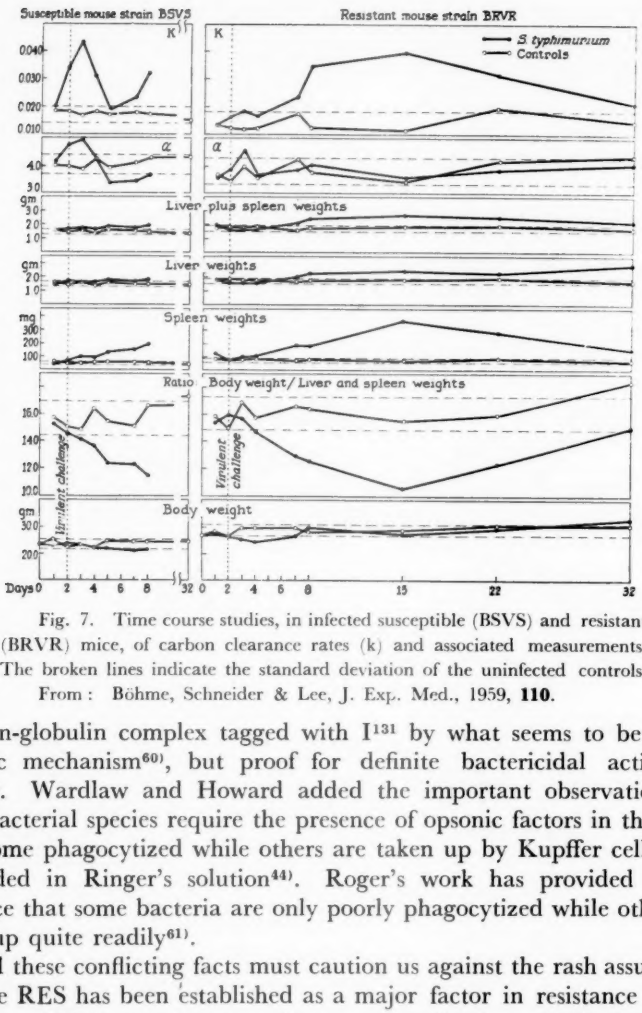

Fig. 7. Time course studies, in infected susceptible (BSVS) and resistant (BRVR) mice, of carbon clearance rates (k) and associated measurements. The broken lines indicate the standard deviation of the uninfected controls. From: Böhme, Schneider & Lee, J. Exp. Med., 1959, **110**.

albumin-globulin complex tagged with I^{131} by what seems to be an enzymatic mechanism[60], but proof for definite bactericidal activity is lacking. Wardlaw and Howard added the important observation that some bacterial species require the presence of opsonic factors in the serum to become phagocytized while others are taken up by Kupffer cells when suspended in Ringer's solution[44]. Roger's work has provided further evidence that some bacteria are only poorly phagocytized while others are taken up quite readily[61].

All these conflicting facts must caution us against the rash assumption that the RES has been established as a major factor in resistance against infection. While previous investigators generally favored this hypothesis the results of more recent experimentation render its validity doubtful. Much remains still to be learned before it will be permitted to say that we have truely advanced beyond the concept drafted by Kiyono, Aschoff and Landau some forty-odd years ago.

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Studies on Experimental Influenza in Mice

I. Virus Growth and immune Response in Mice when the Inoculum was Given from Three Different Routes

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The fact that the route of infection in experimental animals determines the growth characteristics of bacteria in any tissues concerned and consequently the fate of animals too, has already been established.[1] With experimental viral infections, the same phenomena have been noticed particularly when the fate of host animal is taken into account in response to the different route of inoculation. For example, influenza virus is most effective in killing mice when administered intranasally, but after intravenous injection of large dosis of influenza virus, mice usually survive although they are accompanied by viral multiplication and some pulmonary consolidations. The difference thus observed has been explained by different distribution of virus or by the grade of virus multiplication or by the total mass of respiratory epithelium involved.[2]

When we have been dealing with a lot of murine influenzal infections for the other experimental purposes, using three-weeks-old mice, the fact has been noticed that after the intraperitoneal inoculation of large dosis of virus, virus growth in lung was just comparable to that obtained by inhalation of the small inoculum. In spite of such remarkable growth of virus in lung at the time of intraperitoneal inoculation, the mice survived followed by an improvement of the lung lesions. Sudden fall of virus titer in lung observed on the fifth day after infection was a characteristic feature at this occasion. As an early rise of antibody at the time of intraperitoneal injection of influenza virus has been already described,[3][4] the effect of immune mechanisms on the final outcome of virus growth was of particular interest.

Studies to be described in these series papers are derived from the observation along the line written above and the final purpose is to get the information on the participation of immune mechanisms, humoral or

cellular, to the virus growth and to the development of inflammation in animals. This paper chiefly describes the fundamental results obtained in pursuing the growth of virus in several organs on the first, third and fifth day after infection by means of three different inoculation procedures, i.e. intranasal, interacerebral and intraperitoneal. Preliminary studies on antibody responses of these animals against the stimuli given from three routes have been also involved.

Materials and Methods

The virus. The PR8 strain of type A influenza virus was used. It has undergone seven passages in ferrets, 593 in mice, 170 in eggs and again 29 in mice. 10^{-4} broth dilution of the infected lung, obtained two days after infection, was used as the seed inoculum and with 0.2 ml of this material, 11-days old embryonated eggs were infected allantoically. After 48 hrs. of incubation, allantoic fluid was harvested and kept at $-25°C$. This infected fluid was used as the inoculum throughout the experiment and had the 50 per cent egg infectiveity dosis of $10^{9.9}$ and hemagglutinin of $10^{3.7}$ per ml.

The mouse. Three-weeks old white mice ofdd strain weighing 10 grm. from the colony of the own farm were used throughout the investigation.

Mouse inoculation techniques

For the purpose of intranasal inoculation, groups of mice were placed in a 2-liter of chamber and sprayed with an appropriate dilution of infected fluid for a period of 30 minutes. The sprayer employed was a vaponefrin type veporizer and it produced an extremely fine mist, some of which was naturally inhaled by the mice at a pressure sufficient to produce a fog, used 3 ml of fluid during 30 minutes of spraying.

Intracerebral and intraperitoneal inoculations were done as usual.

Method for obtaining bronchial washing

According to the description of Wagner[2], the amount of free virus on the respiratory surface of the tracheo-broncho-alveolar tree has been estimated. 0.5 ml of broth was used for washing and approximately 80 per cent of the broth could be recovered.

Infectivity titrations

Ten per cent suspension (wet weight) of each tissue was emulsified without abrasive in a broth. This material was then diluted in 10 fold step and 0.2 ml aliquots of each were injected into the allantoic cavities of 4 eggs. The 50 per cent infectious dosis (EID_{50}) was calculated using the method of Reed and Muench. In the table, virus amount recalculated on the basis of whole weight of each organ, has been described.

Hemagglutinin titrations

Ten per cent suspension of each organ in a broth was tested for its

content of viral hemagglutinins (HA). After the treatment with receptor destroying enzyme (RDE)[5], non-specific agglutinins have been removed by high-speed centrifugation. When agglutinins were found in any materials tested, the fluid was again tested whether the agglutinations are inhibited by the anti-PR8 rooster serum. Thus all hemagglutinins described in the text are the hemagglutinins of the PR8 virus but not others.

Hemagglutinin inhibition titrations in estimating the antibody titer in the serum was performed after pretreatment of the serum with a cholera filtrate (588). The fundamental approach in removing non-specific inhibitors in serum or organs of mice have already appeared.[5]

Complement fixation test (CFT)

Both soluble (S) and viral (V) antigens were used. S antigen was prepared according to the description of Kirber *et al.*[6] from the infected chorioallantoic membrane of the PR8 virus, followed by differential centrifugation. V antigen consisted usually of elementary body suspension of the PR8 virus which was prepared by differential centrifugation.

Complement fixation technique employed was almost similar to the description of Schmidt and Lennette.[7] Serial 2-fold dilution of antisera were made and distributed in 0.2 ml amounts of three rows of tubes. To each individual dilution series, S and V antigens were added in 0.2 ml volumes and 0.2 ml of guinea pig complement (2 exact units). The third row was served in testing the anticomplementary action of the serum. After incubation at 4°C overnight, 0.5 ml of a one per cent suspension of sensitized sheep red cells were added to each tube and the test was read after further incubation at 37°C for 30 minutes. The reciprocal of the highest serum dilution giving a 3 + fixation was taken as the titer.

Experimental Results

Virus growth in lung, liver, spleen and kidney after inoculations from three different routes

The growth of PR8 virus in these organs have been titrated both by means of EID_{50} and HA, but the growth in a sense of the former will be described first. The purpose of this experiment was two-fold. One is to know whether the extent of virus growth in lung is different with different inoculation procedures. Sedondly, the amount of virus detectable in other organs than lung was to be examined in order to ascertain the concept of pneumotropism with this virus, and also to know the significance of the participations of these organs in antibody production.

For the purpose of intranasal infection, inoculum size which corresponds to the 2 LD_{50} have been introduced into mice. The virus was given by inhalation but not by instillation. The details how to calculate

the absolute amount of virus introduced into the respiratory tract under our standard inhalation procedure will be later described in the second paper[8] but here $10^{3.0}$ EID_{50} was introduced into a mice by inhalation. As a result, this amount of virus killed all of mice, 7–9 days after infection.

For the intraperitoneal and intracerebral inoculation, 0.3 ml of 10 fold dilution of infected allantoic fluid and 0.03 ml of undiluted allantoic fluid was used, respectively. By means of this procedure, $10^{8.4}$ EID_{50} virus was introduced into a mice. All of the mice, however survived.

One of the typical results from three repeated experiments has been illustrated by Fig. 1.

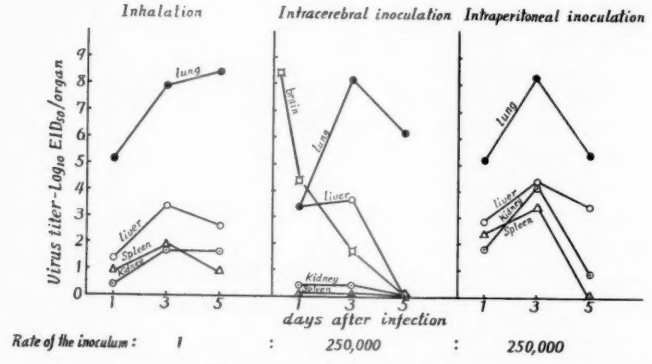


Fig. 1. Virus growth in organs of mice after three different inoculations.

One can notice the highest growth in lung no matter how the virus is introduced. In other words, the organ lung seems to support the best growth of virus among the organs tested, even at the time when virus was not introduced directly from epithelial lining of the bronchus. Further, if one compare the absolute amount of virus in lung, the titer obtained by intranasal inhalation was also obtainable by both intracerebral and intraperitoneal injection, confirmaing the preliminary observation, at least when the three-weeks-old mice have been used. On the third day, the titer reached up to $10^{8.0-8.2}$ EID_{50} per lung. The titer of bronchial washings was $10^{8.3}$, $10^{8.2}$ and $10^{8.2}$ EID_{50}/ml with intranasal, intracerebral and intraperitoneal injections. Thus when the virus titers in lung and bronchial washings are compared on the third day, there was no significant difference between these three inoculations. However, the difference was noticed between these three, when the shape of growth curve was compared. The maximum titer obtained by inhalation did not fall off on the fifth day. On the contrary, when the virus was introduced from unnatural routes, sharp fall was noticed on the fifth day.

In the organs other than lung related growth curve have been obtained after the inoculation of different amount of virus from three different routes, except that of kidney and spleen at the time of intracerebral inoculation, where the significant amount of virus was not found even on the third day. In case of intraperitoneal injection, the growth in these organs reached maxmum usually on the third day and the infectivity of the tissue never exceeded $10^{4.5}$ per organ. The significance of the infectivity detectable in these organs will be discussed in the next chapter.

The nature of virus detectable in each organ with regard to the EID_{50}/HA ratio

Viral hemagglutinins contained in each organ have been also examined using both chicken and guinea pig red cells. Guinea pig cells were used to meet the possibility to detect the virus particle which has the different surface character from that of the original inoculum. Allantoic fluid used as the inoculum agglutinated guinea pig red cells as well as chicken red cells up to the same dilution.

Detectable hemagglutinins have been listed in Table I where 0 means below $10^{1.6}$. When this titer was compared to that of egg infectivity obtained with the same suspension of each organs, EID_{50}/HA (I/H) ratio was calculated and this titer was shown in Table II.

In the lung, reasonable increase of hemagglutinins was obtained when the virus was given intranasally. The I/H ratio was also in the range of established value and this was almost the case with bronchial washings examined on the third day, where a little lower ratio was obtained. By intraperitoneal and intracerebral injections, the highest HA in lung was found on the third day as in the case of EID_{50}, but quite characteristic feature has been noticed when the virus in lung on both first and fifth day was examined with regard to the I/H ratio.

In these cases, I/H ratio was fairly low, particularly with the virus detectable in lungs on the first day after intracerebral inoculation, suggesting the possible distribution of incomplete or inactivated virus particles among the population. Whether the one recognized on the first day has the origin of inoculum virus and that on the fifth day has the origin of grown virus, is not examined so far.

With organs other than lung, hemagglutinins have not been detectable when the small inoculum was given intranasally. But when the large amount of virus was given from unnatural routes, liver and kidney was the organ where hemagglutinins had been detectable. The matter of interest was the hemagglutinin detectable in kidney. Because at the time of intracerebral injection, the infectivity was hardly detectable in kidney, but relatively high hemagglutinins were found on the first and fifth day. This tendency was also true with viruses at the time of intraperitoneal injection where hemagglutinins were detectable on the first and third day. In

TABLE I

Hemagglutinius Detectable in Organs of Mice after 3 Different Inoculations

Inoculation routes	Organs	HA/gr tissue					
		1st		3rd		5th	
		F*	G*	F	G	F	G
Intranasal	Lung	0	0	2.8†	2.5	3.1	3.1
	Liver	0	0	0	0	0	0
	Spleen	0	0	0	0	0	0
	Kidney	0	0	0	0	0	0
	Bronchial washing	n.d.‡	n.d.	3.1	3.1	n.d.	n.d.
Intracerebral	Lung	1.9	1.9	1.9	2.2	1.9	1.9
	Brain	0	0	0	0	0	0
	Liver	0	0	1.9	1.9	2.2	2.5
	Spleen	0	0	0	0	0	0
	Kidney	2.5	2.2	0	0	2.2	2.2
	Bronchial washing	n.d.	n.d.	2.2	2.2	n.d.	n.d.
Intraperitoneal	Lung	1.9	2.2	2.5	2.8	1.9	2.2
	Liver	0	0	2.2	2.2	0	0
	Spleen	0	0	0	0	0	0
	Kidney	2.2	2.5	1.9	1.9	0	0
	Bronchial washing	n.d.	n.d.	3.1	3.1	n.d.	n.d.

* F and G stand for the hemagglutinius obtained by fowl cells and guinea pig cells.
† Titer was expressed as the neg. log to the base 10, where 0 means below 1.6.
‡ not done.

TABLE II

EID_{50}/HA ratio Calculated with Detectable Hemagglutinins

Routes		Inhalation			Intracerebral inoculation			Intraperitoneal inoculation		
Inoculum		$10^{8.0}EID_{50}$			$10^{8.4}EID_{50}$			$10^{8.4}EID_{50}$		
Days of sac.		1st	3rd	5th	1st	3rd	5th	1st	3rd	5th
Organs tested	Lung	n.c.*	6.2	6.4	2.6	7.3	5.3	4.3	6.7	4.6
	Liver	n.c.	n.c.	n.c.	n.c.	2.3	<0†	n.c.	2.8	n.c.
	Kidney	n.c.	n.c.	n.c.	<0	n.c.	<0	0.8	3.3	n.c.
	Bronchial washing		5.2			6.0			5.1	

* n.c. indicates that the value is not able to be calculated because of the lack of HA.
† <0 means that the HA titer exceeds that of EID_{50}.

both cases I/H ratio was quite low.

Hemagglutinins detectable in the liver on the third day by both intracerebral and intraperitoneal injections and on the fifth day by the former had again low I/H ratio.

Hemagglutinin titers obtained with guinea pig red cells were not so different from those obtained with chicken red cells. However, as far as the viral hemagglutinins in lung at the time of both intraperitoneal and intracerebral inoculations is concerned, the virus agglutinated guinea pig cells more than chicken red cells and this tendency was not found at the time of inhalation.

Response of the host in immune mechanism after inoculations from three different routes

Antibody titer detectable in the sera seems to be at least an indication, how the resistance to the re-infection proceeds. Preliminary study was performed to know the antibody level in the serum after three different inoculations. Table III illustrates these results. When a different

TABLE III

Antibody Response of Mice after Inoculations of the PR8 Virus from Three Different Routes

Exp.	Routes	Inoculum size (EID_{50} or HA)	HAI titer of the serum after the day of		
			5	7	10
With the different amount of alive virus	i.n.*	$10^{3.0}$	32	32	n.d.†
	i.c.	$10^{3.4}$	512	1,024	2,048
	i.p.	$10^{3.4}$	512	2,048	1,536
With the same but low amount of alive virus	i.n.	$10^{2.0}$	<16	64	512
	i.c.	$10^{2.0}$	<16	<16	<16
	i.p.	$10^{2.0}$	<16	<16	<16
With the same amount of formaline killed virus	i.n.	$10^{1.3}$HA	<16	<16	<16
	i.c.	$10^{1.3}$HA	512	1,024	2,048
	i.p.	$10^{1.3}$HA	512	512	1,024

* i.n., i.c. and i.p. stand for the intransal, intracerebral and intraperitoneal inoculation, respectively.

† n.d. indicates the experiment was not done because of the death of mice.

amount of alive virus was given just alike in the experiments described above, high antibody titer was detectable on the fifth day after both intracerebral and intraaperitoneal inoculations. After inhalation, however, provable antibody was very poor as far as hemagglutination inhibition antibody was concerned. When low inoculum, such as $10^{2.0}$ EID_{50}, was

given from three different routes, the antibody rise was shown only by inhalation, possibly by the incremental growth of the virus.

Formalin killed virus was also introduced from three routes. In each case absolute amount of 20 HA unit was introduced. This was done because if a large amount of alive virus was given intranasally, mice usually died before 10th day and the antibody resposes after seventh day was not able to be examined for comparative study. The tendency observed by intracerebral or intraperitoneal inoculation of large alive virus was obtained even with formalin killed virus, but there was no antibody rise when a large amount of killed virus was given by intranasal inoculation.

The last observation was confirmed by challenging mice on the 10th day with $100LD_{50}$ virus by intranasal inhalation as has been illustrated in Fig. 2. Defense mechanisms proved after intracerebral and intraperitoneal injections was not obtained after intranasal inoculation of inactivated virus.

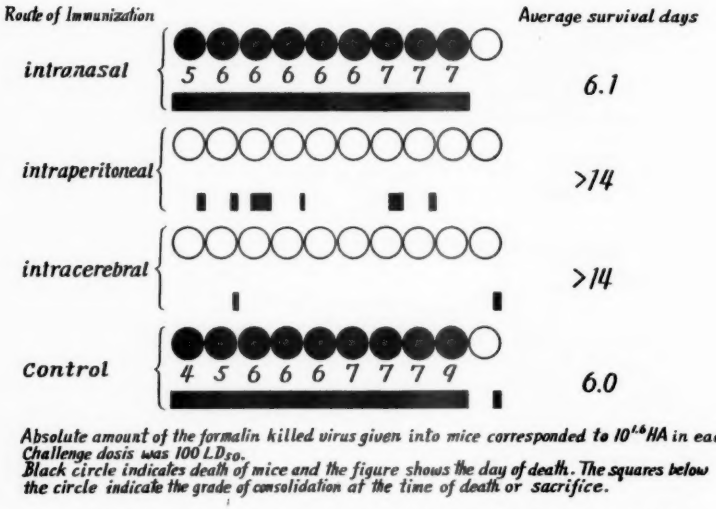

Route of Immunization
Average survival days
intranasal
5 6 6 6 6 6 7 7 7
6.1
intraperitoneal
>14
intracerebral
>14
Control
4 5 6 6 6 7 7 7 9
6.0
Absolute amount of the formalin killed virus given into mice corresponded to 10^4.6 HA in each.
Challenge dosis was 100 LD50.
Black circle indicates death of mice and the figure shows the day of death. The squares below the circle indicate the grade of consolidation at the time of death or sacrifice.

Fig. 2. Result of challenge test done 10 days after the injection of formalin killed virus from three different routes.

Detailed study on the production of antibodies both after intranasal and intraperitoneal inoculations

Different manner in producing antibodies found between the mice receiving intracerebaral or intraperitoneal inoculation on the one hand and intranasal inoculation on the other, has been further examined. On the basis of the observation described above, $10^{8.3}$ EID_{50} virus was given in-

traperitoneally and $10^{2.7}$ EID_{50} alive virus was given intranasally into 70 mice, respectively. After these inoculation, ten mice were killed on the day of choice and the pooled serum was tested for their antibody titer by means of hemagglutination inhibition test and of complement fixation test using both V and S antigens. The typical result was illustrated in Fig. 3 and 4.

Test	Days after inoculation						
	2	3	4	5	7	10	15
HAI	<32	64	512	512	1,024	1,024	2,048
CFT-S	< 8	< 8	< 8	< 8	16	32	⩽32
CFT-V	< 8	8	16	32	256	256	n.d.

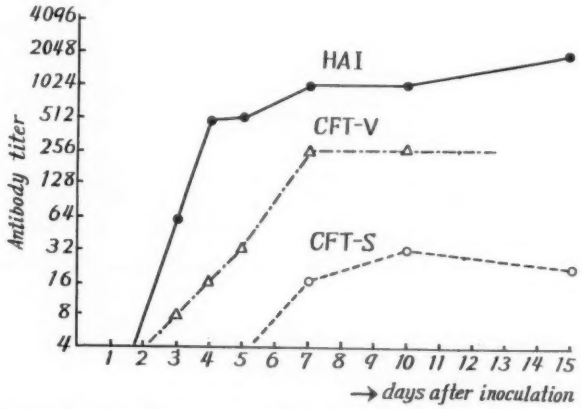


Fig. 3. Antibody response of mice after intraperitoneal inoculation of a large dosis of alive virus.

Early rise of hemagglutination inhibition titer and anti- V titer was evident at the time of intraperitoneal inoculation (Fig. 3). In this case, logarithmic increase of hemagglutianation inhibition antibody was found from the second day up to the fourth day. On the other hand, the antibody rise after intranasal inoculation was first detectable on the seventh day (See, Fig. 4). Thus the early rise of antibody was shown only after intraperi toneal inoculation.

One more effect to be noticed at the time of intraperitoneal inoculation is that the rise of anti-S titer is also followed with the rise of the other two titers. This is evident because when the formalin killed virus was given from the same rout (See, Fig. 5), there was no rise in anti-S titer, in spite of the fairly good respose in both hemagglutiantion inhibition and anti-V

Test	Days after inoculation					
	4	5	7	10	15	20
HAI	<32	<32	128	1,330	2,040	1,410
CFT-S	< 8	< 8	< 8	16	64	32
CFT-V	< 8	< 8	16	128	n.d.	n.d.

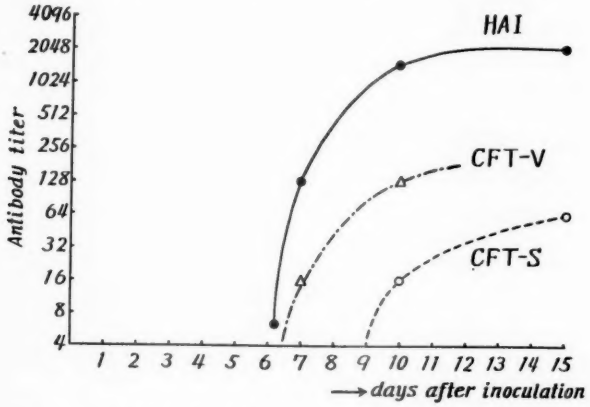


Fig. 4. Antibody response of mice after intranasal inoculation of a small dosis of virus.

titers. The fact seems to support the possible growth of virus in mice when the alive virus was given intraperitoneally.

Early rise of antibody at the time of intraperitoneal inoculation was verified by the challenge test where the virus amount of 1000 LD_{50} was given at 3,5 and 10 days after intraperitoneal inoculation of alive virus. The result shown in Fig. 6 clearly indicates the complet protection of the mice on the fifth day after inoculation and one thirds protection on the third day.

Discussion

Virus growth in lung was compared after intranasal, intracerebral and intraperitoneal inoculations of influenza virus, PR 8, into the three-weeks-old dd mice. The maximum growth which can be expected in the light of many studies done on the similar subject was obtained not only at the time of intranasal inhaltion but also at the time of intracerebral and intraperitoneal inoculation. The observation against this result was obtained recently by Wagner[2], where he got the restricted growth of virus in lung

Test	Days after inoculation						
	2	3	4	5	7	10	15
HAI	<32	32	128	256	340	512	340
CFT-S	< 8	< 8	< 8	< 8	< 8	< 8	n.d.
CFT-T	< 8	<16	8	32	64	64	n.d.

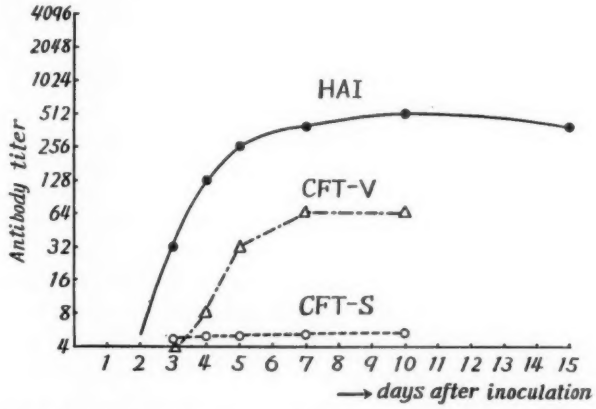
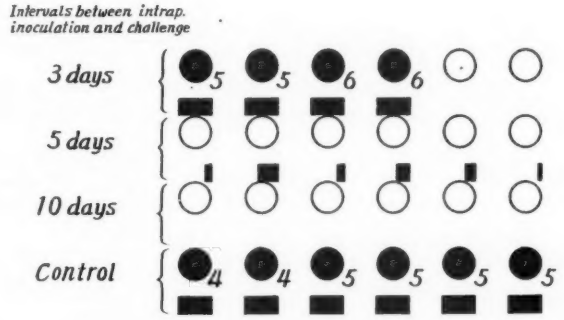


Fig. 5. Antibody response of mice after intraperitoneal inoculation of formaline killed virus.



The details of exp. condition was identical as in the exp. described in Fig. 2.

Fig. 6. Aquired immunity after intraperitoneal inoculation of alive virus.

abter intravenous inoculation into four-weeks-old mice weighting 12–15 g. In our experiment, however, a large amount of virus was found in lung on

the third day after intracerebral and intraperitineal inoculations. The growth of virus in this occasion, seems to be supported by other additional experimental results. Firstly, the virus was found not ouly in lung but also in bronchial washings and the I/H ratio of the fluid suggested the completeness of the particles. Secondly, high anti-S titer appeared when the virus was given intraperitoneally. Thirdly, the analysis of inhibitiors detectable in bronchial wasings revealed a mirror image of the virus growth curve, though the fact was not described here.[10] The age of mice or different route of inoculation probably, will be the clue to explain the difference of maxium titer obtained by Wagner and by us.

However, the growth characteristics of virus in lung after intracerebral and intraperitoneal inoculations was not quite similar when compared to that obtained after inhalation, in respects of the nature of virus found on the first day of inoculation and also of the sudden fall of virus titer on the fifth day.

With regard to the incompleteness of the virus particles found on the first day of both intracerebral and intraperitoneal inoculations, we do not have good explanation yet, and it was particularly the case after intracerebral inoculation. In the third report of the series work,[9] we are going to describe the early distibution of the P^{32} labeled virus after inoculation form three different routes. In that stduy, when the distributiion of the inoculum in lung three hours after inoculation was compared, the activity found in lung after intracerebral inoculation was as low as 2 per cent of that obtained by intranasal inoculation. On the basis of this observation, the low I/H ratio obtained on the first day after intracerebral inculations rather suggest the growth of virus in some unusual manner than the accumulation of inoculum virus.

Histopathological study which will be reported elsewhere seems to support the idea, though does not fully approve the idea. Tissue culture study[11] done with the mouse fibroblast originated from lung tissue revealed the good growth of this virus. The body of evidences seems to suggest that the virus may grow in some cell of the lung other than that of bronchial epithel, but the fact should be examined further in detail.

Sudden fall of virus titer found on the fifth day of unnatural inoculations seems to be correlated with the early appearance of antibody. From the second day up to the fourth day, there was a logarithnic increase of antibody titer. On the fifth day, immune state of mice was perfect in a light of challenge test. Thus the virus titer which decreased on the fifth day seems to be correlated with the acquisition of immunity which succeeds to the virus growth. Did the antibody found in the sera react with free virus which was going to invade the next cell? Did te antibody penetrate the cell and inhibit the protein synthesis of this virus? Did the cellular immu-

nity already established make the further growth of virus impossible? Further analysis of these respects is now going on by one of the authors, and the part of the study will be reported in the seventh paper of the series work.

The last question to be discussed here is the non-infectious hemagglutinins found in kidney and liver. As has been pointed out in the section of materials and methods, the hemagglutinins found was the viral origin but no others. Based upon the known function of these organs, the accumulation of free particles once thrown in the blood stream seems to be conceivable, but we do not have any result to approve this idea. The alterative explanation seems to be the incomplete growth of virus in these organs which will be backed up in tissue culture studies to be reported in the fourth paper.[10]

Summary

Virus growth in organs of mice after intranasal, inracerebral and intraperitoneal inoculations of influenza virus, PR8 was studied.

Even the inoculum was given from unnatural routees, the highest growth was obtained in lung among the organs tested. The mouse here used was three-weeks-old one and the virus titer in lung after intraperitoneal and intracerebral inoculations of large dosis of virus was just comparable to that obtained after inhalation of the small inoculum. In spite of such good growth, mice did not succumb after these unnatural inoculations, and sudden fall of virus titer in the lung was remarkable on the fifth day. Early and high antibody rise at these occasions was correlated to this sudden fall of virus titer.

The significance of hemagglutinisns with low infectivity detectable in kidney and liver particularly at the time of intraperitoneal or intracerebral inculations was discussed.

Acknowledgement

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Studies on Experimental Influenza in Mice

II. Absolute Amount of Virus Introduced into the Respiratory Tract of Mice by Standard Inhalation Procedure

By

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Intranasal instillation procedure have been used customly for infecting mice with influenza viruses. However, to introduce the uniform infection into a large number of mice, atomizing the virus as the mist was rather a prodceure of choice. Many comparative studies of these two procedures have been already described.[1)2)3)]

In our laboratory, though the inhalation procedure had been used for these years[4)], the amount of virus really introduced into the respiratory tract of mice was not exactly known. In developing the experiment, however, it was necessary to get the information of the amount of virus really introduced by inhalation procedure. Distribution of the virus along the naso-tracheo-broncho-alveolar tree was also the matter of interest, particularly in discussing the difference of the two procedures, mentioned above.

Using P^{32} labelled virus, the amount of virus introduced into the respiratory tract after intranasal instilaltion and inhalation of the inoculum and its distribution in the respiratory tree were studied. The result obtained along this line is a subject of this communication together with the procedure of labelling virus. Some comparative observations to approve the advantages of inhalation than instillation in getting uniform infections have been also involved.

Materials and Methods

The virus The PR8 strain of type A influenza virus was used. It has undergone seven passages in ferrets, 593 in mice, 170 in eggs and again 29 in mice. 10^{-4} broth dilution of the infected lung suspension obtained two days after infection, was used as the seed inoculum and with 0.2 ml of this material, 11-days old embryonated eggs were infected. After 48 hrs. incubation, the allantoic fluid was harvested and kept at 25°C. This infected fluid was used throughout the experiment and had the fifty per cent egg infectivity dosis of $10^{9.8}$ and hemagglutinin of $10^{3.7}$ per ml.

Sendai virus[5] was also used. It has undergone 12 passages in mice, one in rat, 3 in mice, 19 in eggs and again 26 in mice. Infected allantoic fluid was obatined just alike in the case of the PR8 virus. This fluid had fifty per cent egg infectivity dosis of $10^{9.7}$ and hemagglutinin of $10^{3.1}$ per ml.

The mouse Three-weeks old white mice (dd strain) weighing 10 g. in average were obtained from the colony of the own farm and used throughout the investigation.

Mouse inoculation techniques

For the purpose of inhalation, groups of mice were placed in a 2-liter chamber and sprayed with the appropriate dilution of infected fluid for a period of 30 minutes. The sprayer emplyed was a vaponefrin type vaporizer and it produced an extremely fine mist, some of which was naturally inhaled by the mice at a pressure sufficient to produce a fog, used 3 ml of fluid during 30 minutes of spraying.

Intranasal instillation was done as usual under ether anestesia and 0.05 ml amount of virus was introduced.

Hemagglutinin titration Salk's pattern method has been emplyed. To the serial two-fold dilution of the tested material in 0.5 ml, 0.5 ml of 0.5% chicken red cell suspension was added and the results were read after standing for 1 hr at 10°C.

Preparation of P^{32} labeled virus

P^{32} was obtained from the radiochemical center, Amersham, England in dilute hydrochloric acid. Other technical details in preparing the tracer virus were described in the text.

Determination of the radioactivity with various organs of mice

Usually 10 mice were used for one determination, unless otherwise noted. The mice were first killed by bleeding under light ether anaesthesia. Organs to be tested were removed and washed five times in phosphate buffered saline (PBS,M/100) and blotted. For the purpose of wet oxidation, nitric acid was used. After the completion of the oxidation total 1 or 2 ml of samples were placed in stainless steel cupped planchets and dried under infra-red lamp. Counts were made with a mica end window Geigher-Müller tube and an ALOKA A DC-1 type of 1000 scaller. Counts per minute (cpm) found with each specimen were recalculated on the basis of the total amount of oxidized fluid and shown in the figure of the text as an absolute value found in each organ.

Experimental

The amount of virus introduced by inhalation calculated on the basis of comparing the dilution extent of the inoculum which will kill the mice by means of inhalation with that obtained by means of instillation

The data on the mortality of mice both by instillation and inhalation

of the same stock Sendai virus were available from experiments of other purposes. Fig. 1. illustrates these results and the regularity of death tendancy by inhalation is apparent when the accumlated death rate obtained from repeated experiments performed on different day was plotted against the days after infection(upper part of the figure). This was not the case at the time of instillation. In addition to this difference, dilution effect of the virus suspension as inoculum on the fate of mice was quite different

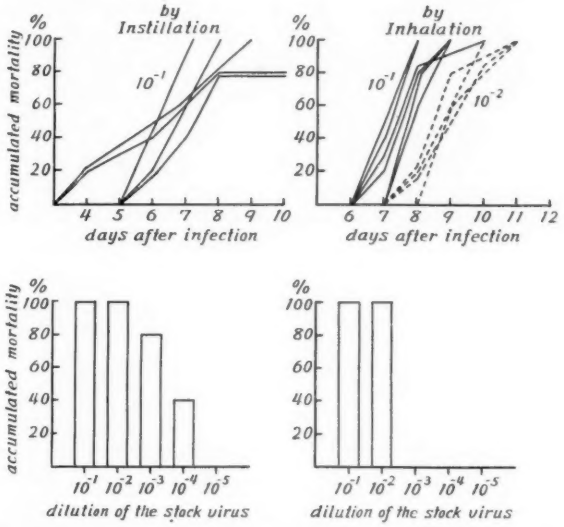

Fig. 1. Mortality of mice after two different intranasal inoculations of the Sendai virus.

Solid and broken lines in the upper figure denote the 10^{-1} and 10^{-2} dilution of stock virus, respectively.

between these two procedures (lower part of the figure). At the time of instillation, 10^{-3} diluted virus killed 80 per cent and 10^{-4} diluted one killed 40 percent of the mice. With inhalation, however, 10^{-2} killed the total mice but 10^{-3} did not kill any of them. Thus the phenomenon of all or nothing seems to exsist with the latter procedure but not with the former. This fact is the second feature to be considered as an essential difference found between two procedures. Having these kind of differences in mind, one can calculate the LD_{50} obtained by these two procedures. One of the typical experiments are illustrated by Table 1 both with Sendai and the PR8 strain of influenza virus.

With Sendai virus, the same stock virus gave the LD_{50} titer of 3.7 at the time of instillation but at the time of inhalation the titer was 2.5.

TABLE I

The Comparison of Death Tendency obtained by Inhalation of Both Sendai and PR8 Viruses with that by Instillation

Dilution of the stock virus	Sendai virus (allantoic fluid, $10^{9.7}$ EID_{50}) by Instillation	Sendai virus (allantoic fluid, $10^{9.7}$ EID_{50}) by Inhalation	PR8 (lung suspension, $10^{10.2}$ EID_{50}) by Instillation	PR8 (lung suspension, $10^{10.2}$ EID_{50}) by Inhalation
10^{-1}	D D D D D 6 6 7 7 7*	D D D D D 8 8 8 8 9		
10^{-2}	D D D D D 6 7 7 8 9	D D D D D 8 9 9 9 10		D D D D D D 5 5 5 6 6 6
10^{-3}	D D D D S 7 8 9 9	S S S S S		D D D D D D 6 7 7 7 8 8
10^{-4}	D D S S S 7 9	S S S S S	D D D D D D 5 5 5 6 7 7	D D D D D D 9 10 11 11 11 11
10^{-5}	S S S S S		D D D D D S 7 7 7 8 8	S S S S S S
10^{-6}			D D S S S S 7 9	
10^{-7}			S S S S S S	
LD_{50} calculated	3.7	2.5	5.6	4.5
difference	1.2		1.1	

* Number denotes the day of death after inoculation.

The difference was 1.2. In other words, if we assume that the infection of susceptible cells have been occurred in the same manner with these two procedures, 1/16 volume (corresponds to negative log 1.2 to the base 10) of virus suspension should be inhaled when compared with the 0.05 ml. amount given by instillation. Almost the same value was obtained with the PR8 virus where the ratio was almost 1:13 instead of 1:16., as was shown on the right side of the Table. One more difference found between two procedures which should be taken into account is that when a certain dilution of virus suspension was given by inhalation, the mice died almost on the same day and this was not the case at the time of instillation. Both Fig. 1 and Table I suggest this tendency.

Preparation of P^{32} labeled virus

The experiment described above may suggest the amount of virus introduced by our standard inhalation procedure, but the exact amount of virus introduced into the respiratory tract had to be verified in developing our experiment. For this purpose, counting of the radioactivity after inoculation of P^{32} labeled virus was rather a procedure of choice than titration by infectivity in eggs, because the early disintegration of the latter

activity had been already established when the virus once penetrates into the susceptible cell.

After the repetition of many preliminary experiments, standard procedures have been established to purify the labeled virus which had the high radioactivity per hemagglutinin and not contaminated with phosphorus of non-viral origin.[6][7][8]

P^{32} in a form of orthophosphoric acid in dilute hydrochloric acid was once heated at 60°C for five minutes in order to remove the impurities other than orthophosphate[9] and then neutralized with sodium hydroxide. 300 μc in 0.2 ml. was injected into allantoic cavities of 10-day old embryonated eggs. Four hours later, standard inoculation of the PR8 virus was made and the eggs were incubated for further 48 hrs. Then the eggs were chilled and allantoic fluids were collected. Usually ten eggs were employed at the same time. The allantoic fluid thus obtained was used for further purification (Fig. 2). After light centrifugation, clear supernatant was obtained and 10 per cent chicken red cell suspension was added to this supernate in 1/10 volume. These mixtures were left at 4°C for 15 minutes with occasional shaking and then the cells were collected by centrifugation. Sedimented cells were washed four times with PBS at cold and then the virus was eluted at 37°C with PBS of one half the volume of the original fluid. The eluates, thus obtained, were combined and dialyzed against 20 volumes of PBS with repeated changes. With this material, the whole purification procedure described above was repeated once more. However, the elution from the chicken red cells was made in one tenth volume of the original fluid, this time. After dialysis, the preparation was used for the experiment.

Purity of this labeled virus was evaluated by three means according to the description of Hoyle[7] and obtained results were shown in Table II. The first procedure was the adsorption to red cells. The radio-

TABLE II

Purity of the Labeled Virus

Examination procedures	Radioactivity (cpm) of		Per cent left in the supernate	HA left in the supernate	Purity (per cent)
	Starting material	Supernate			
1. Adsorption onto red cells	2,554	146	5.6	8	94.4
2. Ultracentrifugation	2,584	162	6.2	4	93.8
3. Chemical precipitation	1,292	78	6.0	4	94.0

* Specific activity of starting material was 12,920 cpm/5,120 HAU (2.52).

activity left in the supernatant was considered as impurities. The second procedure was the centrifugation at 26,000 g. for 60 minutes. Here again

the radioactivity found in the supernatant solution was taken as impurities. The third procedure applied here was the precipitation of the virus by means of salting out with half saturated ammonium sulfate. After centrifugation at 6,000 g. for 60 minutes, the detectable radioactivity in the supernate was thought as the contaminant. Average 94 per cent purity was proved by means of these analyses.

Though the analyses of the purity described above do not fully exclude the possible existence of the contaminant P^{32} other than virus,[10] fairly purified virus was thus obtainable. Relative specific activity of the hemagglutinin (cpm/HA) was usually in the range from 2.1 to 2.5.

Distribution of the tracer virus along the respiratory tract after instillation and inhalation

Twenty mice were used for this purpose. Half of them received the instillation of 0.05 ml. amount of tracer virus under light ether anaesthesia. The other half was infected by inhalation under the standard procedure. All of the mice were sacrificed immediately after the inoculations and the whole respiratory tract was taken out as quick as possible and was divided into three parts. To do this, first the skin covering these areas has been cut median and turned over to the lateral side. Mandibular area was excised by scissors and the nasal area including nasal bone, upper plate and epiglottis was taken together with muscles around them and used as the nasopharyngeal part of the tract. The second part, bronchial tree, included all visible tract from pharynx to its entrance into lung tissue. It was resected easily. The rest, i.e. five lobes of lung were used as the third part. Three parts thus obtained from each mouse were pooled in each and after the wet oxidation, radioactivities have been estimated respectively.

The experiment described above was repeated 2 times with the same labeled virus. Each of two experiments gave almost identical result and averaged value on the distribution of this activity into the three parts was illustrated by Table III. In the table, the result of one experiment performed with free P^{32} solutions instead of tracer virus was also illustrated. Here the specimens obtained from 10 mice was titrated their specific activity in each, and the mean value was calculated. In inspecting the result, total 55 per cent of introduced virus was found in the naso-tracheo-bronchoalveolar tree at the time of instillation. Thus the almost half amount of theoretically introduced virus was lost somewhere or at least was not detectable at the time when these parts are removed from mice. By means of inhalation, 6 per cent equivalent have been proved in the total respeiratory tract, on the basis of theoretically instilled virus amount.

If we compare the distribution of the virus into the three part of the tract, almost similar result was obtained with the two procedures. Around

TABLE III

Distribution of Radioactivity along the Respiratory Tract after Inoculation of the Labeled Virus and Free P^{32} by means of Instillation and Inhalation

Inoculation method	Tested respiratory tissues	P^{32} labeled PRS virus				Aqueous solution of P^{32}			
		Counts used	Counts found	Total counts found	% total	Counts used	Counts found	Total counts found	% total
Instillation (0.5 ml)	Nasal tissue	660 cpm per 10 mice	133*	366 (55%)†	36	12,580 cpm per mouse	1,358‡	3,447 (27%)†	39
	Tracheal tissue		57		16		246		7
	Lung tissue		176		48		1,843		54
Inhalation (for 30mins.)	Nasal tissue	1320 cpm/ml fluid was put in nebulizer	17	36 (6%)†	44	251,600 cpm/ml fluid was put in nebulizer	64	180 (1.5%)†	36
	Tracheal tissue		4		10		14		8
	Lung tissue		18		46		102		56

* The value in this column was obtained from the pooled material of total 10 mice.
† Calculated on the basis of count theoretically introduced at the time of instillation.
‡ Average value obtained from 10 calculations was described in this column.

the half of the detectable virus along respiratory tract was in the lung and the rest remained in the upper part.

When the count detectable in the lung by inhalation was compared with that of instillation, around 10 per cent of the latter (18:176) was found in the former. This value was also found when the total amount of virus detectable in the respiratory tract was compared (39:366). Thus the ratio was fairly in good agreement with that calculated on the basis of first experiment, where the dilution extent of virus stock corresponding to the lethal amount was examined. In the first experiment described above the ratio 1:16 was obtained with Sendai virus and 1:13 with PR8 virus, where in this experiment the value was 1:10 with PR8 virus.

The experiment done with free P^{32} gave similar but somewhat different result. In this case, two thirds of the given activity was lost at the time of instillation and only 1% was detectable at the time of inhalation. The difference from that obtained with tracer virus might be explainable by the early escape of free P^{32} from the tract. This was not the case with the virus, as will be reported elsewhere.[11] Distribution in three parts, however, were not quite different with that obtained by tracer virus. The aforementioned ratio calculated with the lung was 1:18 in this case.

Technical reliability of inhalation procedure when compared to the instillation procedure with regard to the uniformity of inoculations

In the above experiment free P^{32} gave not quite different result with

TABLE IV

Deviation of the Detectable Count from Mice to Mice after Introduction of Free P^{32} by Means of Both Instillation and Inhalation

Mouse No.	cpm found in lung after	
	Instillation	Inhalation
1	100	250
2	2,560	140
3	220	250
4	110	340
5	5,580	180
6	690	170
7	1,950	240
8	4,010	200
9	2,280	190
10	110	150
$\bar{x}\pm\mu$	1,761±1895	211±60.1

that of tracer virus, in its distribution into respiratory tract. For this reason, the deviation of the detectable count in the lung from mouse to mouse was examined using free P^{32} solution after two inoculation procedures. Ten mice were employed for each. From the radioactivity of each lung, the reliability of these two procedures has been examined. As it is apparent in Table IV, just a little deviation of the detectable count was found between the mice given free P^{32} by inhalation. However, deviation of the count between each instilled mouse was fairly large than expected.

In this experiment the afore-mentioned ratio of 1:8 was obtained.

DISCUSSION

In determining the amount of virus introduced into the lung by means of standard inhalation procedure, two different approaches have been undertaken. As a result, the ratio of the introduced amount of virus obtained by inhalation to that of instillation was calculated, at least within a range of certain deviation.

First approach undertaken was to determine the dilution extent of the stock virus, which will kill the 50 per cent of mice by means of two procedures. The afore-mentioned ratio was calculated as 1:16 with Sendai virus and as 1:13 with PR8 virus. Further, in the additional experiments performed afterwards, the ratio of 1:13 and 1:10 was again

obtainable with the PR8 virus. Thus the ratio were all within a range between 1:10 and 1:16.

Second approach involved here was the determination of the radioactivity of the lung after the introduction of P^{32} labelled virus by means of two procedures. The ratio 1:10 was obtained in the experiment described here. Thus the ratio obtained by the two approaches were at least within a range of 1:10 and 1:16. However, one have to notice the difference in calculating the ratio between two approaches. In the former approach, the data may reflect the amount of sussceptible cells infected along any part of the respiratory tract, and in the latter approach, the count in the lung was our basis of calculation. In the latter appraoch, however, when the total count in the respiratory tract but not lung was compared, again the same ratio 1:10 was calculated. Thus the results seem to allow us to compute the amount of virus given by inhalation. At the time of instillation, 50% of the 0.05 ml. inoculum was found in the total respiratory tract. At the time of inhalation, average 1:12.5 of the virus was detectable in the lung when compared to the value obtained by instillation. Thus the amount given by inhalation will be calculated as follows: $0.05\ \text{ml} \times 1/2 \times 1/12.5$, then it will give us the answer of 1/500 ml. This is the amount of solution inhaled to mice within 30 minutes under our standard condition.

Using free P^{32} the ratio of 1:18 and 1:8 was obtained as was already shown in the text. This value was also in the vicinities of the aforementioned ratio obtained with the virus, supporting the above calculation.

In discussing the deviation of the ratio thus calculated, one have to notice the deviation of the amount of virus found in each mouse at the time of instillation of free P^{32}. Because this value was the denominator in calculating the ratio here concerned.

One more method to estimate the real amount of virus introduced into respiratory tract by inhalation procedure, is naturally the comparison of real count found in the total tract with that put in nebulizer. From the results shown in Table III, the value 1/340 ml. (3.9/1,320) was obtained. Unfortunately, this kind of experiment was not repeated several times, thus we have given the value of 1/500 ml. on the basis of whole experimental results.

As far as the distribution of the inoculum into the three parts of respiratory tract is concerned, there was no significant difference between two procedures. The participation of virus distributed in the nasopharyngeal parts to infection, however, is not yet known.

The difference in the death tendency of mice between two inoculation procedures may be conceivable from the variable amount of virus found with each mouse at the time of instillation. In this case, the mortality

curve against incubation period was not always reproducible from experiment to experiment, the day of death was different from mice to mice inoculated with the same amount of virus, and " all or nothing " law did not exist on mortality tendency with regard to the extent of the dilution. In the light of result on the deviation of detectable count at the time of instillation, the disadvantage found in this case seems to be explainable. Thus inhalation procedure seems to be prepferable in getting more uniform state of infection than instillation.

Summary

The amount of virus introduced into the respiratory tract of mouse by our standard inhalation procedure has been calculated on the basis of two experiments.

Firstly, the dilution extent of virus suspensions which will kill the 50% of mice by inhalation was compared with that obtained by instillation. The result indicated that 1/10 to 1/16 amount of virus was effective at the time of inhalation when compared to the value obtained at the time of instillation.

Secondly, P^{32} labeled virus was prepared and the actual counts found along the whole respiratory tract and also in the lung were compared between two groups of mice infected by these two procedures. Here the value 1/10 was obtainable.

Further it was shown that by the procedure of instillation, around 50 per cent of the theoretically introduced amount of virus was detectable in the naso-tracheo-broncho-alveolar tree. On the basis of these figures it was concluded that 1/500 ml. of virus solution which was put into the vaporizer was inhaled in the respiratory tract of mouse by means of our standard inhalation procedure.

Uniform distribution of virus among animals has been suggested by inhalation contrary to the fairly deviated distribution of the inoculum from animal to animal at the time of instillation.

From this observation, the difference of death tendency observed between two procedures have been elucidated.

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Studies on Experimental Influenza in Mice

III. Early Distribution of P^{32} Labeled Virus in Organs of Mice after Administration from Three Different Routes

By

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In the first report of this series work,[1] the growth of virus in several organs of mice was studied when the virus was given from three different routes, i.e. after intranasal, intracerebral and intraperitoneal inoculations. Predominant growth of virus in the lung, no matter how the virus was introduced, has been proved. The amount of virus detectable in the lung on the first day of infection, however, was different between these three inoculations. The highest titer in the lung was found after the inhalation of $10^{3.0}$ EID_{50} virus. Though the larger amount of virus, $10^{8.4}$ EID_{50}, was given both intraperitoneally or intracerebrally, the virus amount in the lung was lower on the first day when compared to the titer obtained after inhalation of the small inoculum. The result may suggest that the known pneumotropism of this virus is not determined by its specific adsorption to the cells involved in lung tissue during its circulation in the blood stream but should be understood that the cells in lung tissue have the full activity to allow the growth of this virus. However, to confirm the above idea and also in developing the experiment, exact knowledge on the distribution of the virus at the early site of inoculation was necessary.

This was done by using the highly purified P^{32} labeled virus. Distribution of this virus in organs of mice both 30 minutes and three hours after infection from three different routes has been examined by means of radioactivity. Facts presented here were concordant with the above idea and some dynamic aspect of the virus distribution at the early stage of infection was also presented.

Materials and Methods

Preparation of P^{32} labeled virus

The PR8 strain of influenza A virus was used throughout the experiment. The methods employed for virus assays and for preparation of labeled virus have been described already.[2]

Virus inoculation procedures

The dd strain of white mice weighing 10 gr. in average was used. Intranasal instillation, intracerebral and intraperitoneal injection were employed for inoculations. At the time of former two inoculations, 0.03 ml. aliquot of labeled virus was introduced, where at the time of intraperitoneal inoculation, 0.3 ml. of 10 fold diluted virus was introduced. Thus the same activity was introduced into each mouse. As has been pointed out in a previous report,[2] inhalation should be a procedure of choice when compeared to the instillation, in getting uniform infections. But in order to find out large amount of radioactivity in organs of mice, instillation procedure has to be employed in this study.

Determination of the radioactivity with various organs of mice

Ten mice were used for one determination. The mice were first killed by bleeding under light ether aneasthesia. Organs to be tested were removed as usual, washed several times in phosphate buffered saline, blotted and weighed.

For the purpose of wet oxidation, nitric acid was used. After the completion of the oxidation, usually 1 ml. samples were placed in stainless steel cupped planchets and dried under infra-red lamp. Counts were made with a mica end window Geiger Müller tube and ALOKA A DC-I type of 100 scaler. Count per minute (cpm) found with each specimen was recaluculated on the basis of the total amount of oxidized fluid and shown in the figure as an absolute value found in each organ.

Experimental Results

Distribution of radioactivity in brain, trachea, lung, liver, kidney and spleen after three different inoculations of labeled virus

Early distribution of the virus in these organs of mice when the inoculum was given from three different routes, i.e. intranasally, intracerebrally and intraperitoneally, has been studied. For this purpose, radioactive virus was used as the inoculum and the counts detectable in the organs to be tested, i.e. brain, trachea, lung, liver, kidney and spleen both 30 minutes and 3 hrs. after inoculation have been examined. The experiment was repeated three times using 3 different lots of labeled preparation. Table 1 illustrates the property of the virus used. Except the first preparation, the purity of the preparation was analyzed as had been already reported in a previous paper.[2]

At least 2 preparations among 3 are proved to be not contaminated more than 5% of phosphorus other than virus. Though the first preparation was not tested for its purity in the same manner as the remainings, the same purification procedure was employed with all of them.

Distribution of the radioactivity in organs of mice has been shown

TABLE I

Property of P^{32} Labeled Virus

No. of preparations	cpm/ml	HA/ml*	cpm/HA	Purity†
1	1,300	1,280	1.01	not done
2	10,820	5,120	2.11	96.6%
3	12,920	5,120	2.55	95.6-96.2%

* HA stands for hemagglutination unit.

† Determined by chicken RBC adsorption, ultracentrifugation and precipitation with half saturated ammonium sulfate.[2)]

TABLE II

Distribution of Labeled PR8 Virus in Mice after Inoculation from Three Different Routes

Inoculation*			Radioactivity per cent found in					
Preparation No. of virus	After infection	Route†	Brain	Trach.	Lung	Liver	Kidney	Spleen
1	30 minutes	i.c.	16.2	0	1.8	14.4	0.8	1.1
2			16.3	0	1.0	11.0	0.5	0.3
3			16.4	0	0.3	7.5	0.6	0.6
1		i.n.	1.4	1.4	28.8	9.0	1.1	2.0
2			0	1.8	25.5	4.8	0.5	1.0
3			0	2.0	22.2	0.6	0	0.1
1		i.p.	0	0.9	0.4	1.6	0	0
2			0	0.8	0.8	3.5	0.3	0.8
3			0	2.2	1.2	5.1	0.6	1.6
1	3 hrs	i.c.	12.5	0.4	0	8.3	0	0.4
2			10.0	0.2	0.3	9.0	0.4	0.3
3			7.2	0	0.6	9.5	0.4	0.3
1		i.n.	0	0.4	26.7	0.4	0	0
2			0	0.7	23.5	1.3	0.5	0.4
3			0	0.9	20.1	2.1	0.7	0.4
1		i.p.	0.4	0.4	0.4	5.1	1.6	1.4
2			0	0.6	0.6	6.0	1.6	1.8
3			0	0.8	0.7	6.0	1.6	2.2

* In each experiment, 0.03 ml. of the labeled preparation was inoculated intranasally or intracerebrally and 0.3 ml. of 10 fold diluted preparation intraperitoneally. Counts were obtained after wet oxidation of each organ and the per cent was calculated on the basis of the absolute amount of the inoculum.

† i.c., i.n. and i.p. stand for intracerebral, intranasal and intraperitoneal inoculations, respectively.

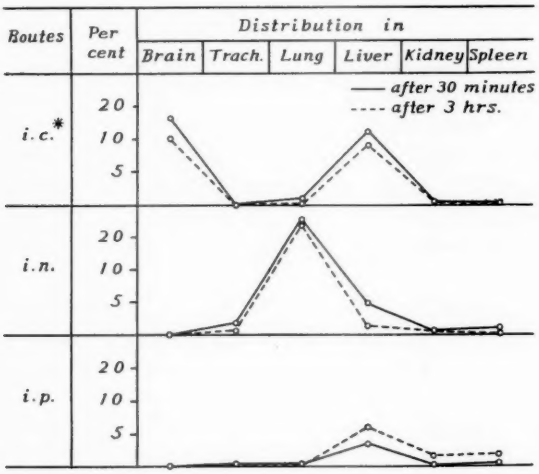
in Table II as a per cent on the basis of total count inoculated. Table III illustrates the actual count obtained in one of the experiments and Fig. 1. is the graphic presentation of this result. Technical details to do

TABLE III

Actual Counts Found in the Organs of Mice after the Inoculations of the Preparation No. 2

Inoculation*		cpm/total organ					
hrs. after infection	Routes	Brain	Trach.	Lung	Liver	Kidney	Spleen
0.5	i.c.	528	0	33	356	16	26
	i.n.	0	58	826	155	16	33
	i.p.	0	26	27	113	9	26
3	i.c.	325	6	9	291	12	9
	i.n.	0	22	761	42	16	12
	i.p.	0	19	19	196	51	58

* i.c., i.n. and i.p. stand for intracerebral, intranasal and intraperitoneal inoculations.



* i.c., i.n. and i.p. stand for intracerebral, intranasal and intraperitoneal inoculations.

Fig. 1. Per centage distribution of the radioactivity in organs of mice after inoculation of labeled virus from three different routes.

this have been already shown in the section of materials and methods.

It will be noted first that the fairly large per cent of the inoculum was detectable in the organs near to the site of inoculations. At the time of intranasal instillation, as high as 25 per cent of the inoculum was detectable in the lung both after 30 minutes and 3 hrs. This result was also in good agreement with the results obtained in a previous paper,[2] in which the activity was measured immediately after the inoculation.

At the time of intracerebral inoculation, 16 per cent of the inoculum was detectable in the brain at 30 minutes but the amount decreased at 3 hrs. The latter titer did not agree well when the count of 3 experiments are comparred, but the fact was not examined further. At the time of intracerebral inoculation, high radioactivity was also found in the liver next to the brain, suggesting the early escape of the particle into the blood stream.

When the virus was introduced intraperitoneally, the total amount of radioactivity detectable in the organs was fairly low when compared to the results obtained by two other inoculations, probably owing to the slow penetration of the virus from the peritoneal cavity. In spite of the fact, the liver was the place where the largest count was detectable. In this connection, it will be noted that when the count of 30 minutes and 3 hrs. are compared, apparent increase was obtainable in the liver. This kind of increase was found only in this case and seems to support the idea of the slow penetration of the virus from the peritoneal cavity.

Second conclusion from the inspection of Table II is that the liver is the place where virus particle once thrown into the blood stream are collected, and this was not the case with lung. In general, liver is known to contain many scavenger cells and these cells are active particularly when the particles are introduced into a blood stream.[3] At any rate, counts detectable in trachea and lung after intracerebral and intraperitoneal inoculations were lower when compared to that obtained after instillation. This was particularly the case with lung.

Low but significant counts were also detectable in kidney and spleen in almost all occasions but the consistent titer was found only at three hours after intraperitoneal inoculation. This result was very concordant with the result obtained in the first report,[1] where the distribution of virus in liver, spleen and kidney after the inoculations from three different routes was examined by means of infectivity on the first day after infection. In that experiment, remarkable infectivity was found in liver, spleen and kidney in this order at the time of intraperitoneal injection and in liver at the time of intracerebral inoculation. In the latter occasion, however, infectivity was not found in kidney and spleen. At the time of instillation, such a high infectivity was not found in liver, kidney and spleen but among them, the titer was highest in liver. These results are all in good agreement with the results of radioactivity obtained here though the experiment was made this time at three hours after infection.

Radioactivity found in blood after intranasal instillation and intraperitoneal inoculations

Wide distribution of the radioactivity in organs such as liver, kidney and spleen even at the time when the virus was introduced directly from

the respiratory tract may suggest that the distribution of virus via blood stream may eventually follow. This was examined by testing the radioactivity of blood after intranasal instillation and intraperitoneal inoculation of tracer virus. After intranasal and intraperitoneal injection of the same absolute amount of tracer virus, 10 mice were killed at 10,30 minutes, one, two, three, four and five hours after the infection, respectively and the citrated blood specimens were tested for their radioactivity. The count obtained with 6 ml. of blood was shown in Fig. 2. Low but significant activity was found from 10 minutes at the time of instillation, and

Route	Hrs. after inoculation						
	10 min.	30 min.	1 hr.	2 hrs.	3 hrs.	4 hrs.	5 hrs.
Instillation	12*	30	15	24	30	210	75
Intraperitoneal inoculation	0	0	18	9	18	48	36

* cpm/6 ml of blood was illustrated.

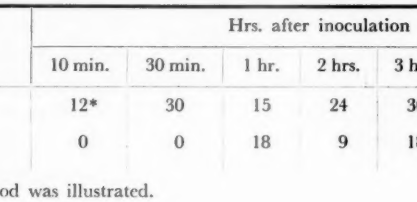


Fig. 2. Radioactivity of blood after instillation and intraperitoneal inoculutions.

from one hour at the time of intraperitoneal inoculation. Thus the results seem to suggest the trickling of the virus into blood stream. Four hours after infection, there was a sudden rise of the count in the blood and the rise was more remarkable at the time of intranasal instillation. Incubation period for this virus growth, in any tissue concerned, is already known to be around 4 hrs. Thus the count detectable here, many reflect a breakdown of the viral phosphorus. Chemical examination of this activity, however, was left for further studies.

Discussion

Current thought holds that there is a phase in virus infections during which disruption of the infected cells results in a loss of viral infectious activity. In accordance with this concept, virus particles which reached

to some organs of mice should not be exactly estimated, when the egg infectivity was employed in measuring number of particles. On the other hand, the counting of radioactivity also may not fully indicate the exact amount of virus particles because of the possibility of its breakdown into nonviral P. However, when the examination was made within a short time after infection, the counting results may fairly reliable in estimating the amount of virus in organs. Moreover, exactness and easiness of the radioactive counting when compared to the egg infectivity titration is in a favour of the former procedure. For this reason the first event occurring in mice organs after the infection have been pursued by means of radioactivity but not of infectivity.

The result in favour of the above thought was obtained that there was no significant difference of count when the 30 minutes activity of the lung was compared to that of 3 hrs. However, 3 hrs. may be the critical point of the estimation. Because the sudden rise of the radioactivity found in blood stream at the 4th hour after infection seems to suggest the break-down of the virus particle.

No specific distribution of the virus into the lung tissue was obtainable at the time of intraperitoneal and intracerebral inoculation of virus into mice. The data together with the infectivity titer obtained with the lung on the first day of infection,[1] suggest that the pneumotropism of this virus should be understood as the ability of lung tissue in allowing the growth of this virus, but not as the specific affinity of particles in its distribution to the cells involved in lung tissues. Using labeled virus, some reports have already appeared on the growth characteristics of this virus in a population of the same cell[4] but the analysis on the distribution or the growth of virus in vivo have never been studied. Development of this work seems to be promissing, particularly in understanding the growth of virus in tissues other than lung and also in analysing the difference of virus growth in lung after natural or unnatural inoculations.

Summary

The same amount of P^{32} labeled influenza virus, PR8, was given to mice by intranasal instillation, intracerebral and intraperitoneal inoculations and the radioactivity of the six organs, i.e. brain, trachea, lung, liver, kidney and spleen was determined both 30 minutes and 3 hours after infection. The count detectable in lung was highest at the time of instillation, but the high distribution of the virus in the lung tissue was not the case, after intracerebral and intraperitoneal inoculations. At the time of intracerebral inoculation, radioactivity was highest in brain decreasing from 30 minutes to 3 hrs. and next in liver. At the time of intraperitoneal inoculation, the total detectable count was low when com-

pared to the activity obtained by other procedures. Here, highest distribution was in the liver.

The virus amount found in several organs was almost consistent when 30 minutes activity was compared to that of 3 hours, except in the case of intraperitoneal injection where the titer shift was found in liver, probably owing to the slow penetration of the virus from peritoneal cavity.

The radioactivities of liver, kidney and spleen at 3 hours were in good agreement with the infectivity found on the first day of the infection.

Sudden rise of P^{32} activity in the blood was noticed at 4 hrs. after intranasal instillation, and the fact was explained as the breakdown of virus particles.

The concept pneumotropism of influenza virus has been discussed from the results obtained in this study.

Acknowledgement

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On Carcinoma of the Gallbladder with Special Reference to the Relation with Calculous or Inflammatory Diseases

By

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It is well known that the carcinoma of the gallbladder is not unusually accompanied with calculi. In fact, we often observed such a concurrence.

Besides, we also experienced some cases which had been cholecystectomized for calculous diseases and in which the gross or, in some instances, the microscopic studies of the open gallbladder revealed the co-existence of carcinoma.

These facts suggest some relations between carcinoma of the gallbladder on one hand and calculous or inflammatory diseases of the same organ on the other hand. To clarify this problem, thorough examinations were carried out in 263 gallbladders which had been extirpated for various surgical disorders.

Experimental and Comments

I. Objects

The objects of this study were 263 gallbladders ectomized in our clinic during the last 17 years for various cholecystic disorders, items of which are shown in Table I.

II. Carcinoma Cases

Among 24 carcinoma cases, tumors could be recognized during operation in 17 cases, while in the remaining seven, examinations of the gallbladder ectomized for calculous or inflammatory diseases found first the concurrence of carcinomatous lesions.

Twenty four carcinomas of the gallbladders are classified by their gross forms, according to Hanser,[1] as shown in Table II. These comprised three cases of the pedunculated papillary type (Fig. 1), three cases of the broad-based papillary type (Fig. 2), five of the nodular type (Fig. 3) and 13 of the diffusely infiltrating type (Figs. 4 and 5).

TABLE I

Items of Cholecystic Disorders Operated in Our Clinic

Neoplasms		26
Carcinoma	24	
Adenoma	1	
Myoma	1	
Calculous diseases		168
Gallbladder	93	
Choledochus	42	
Gallbladder and choledochus	33	
Non-calculous disorders		69
Cholecystitis	31	
Non-inflammatory cholecystopathy	38	
Total		263

TABLE II

Classification of Carcinoma of Gallbladder by Gross Forms (Hanser)

Papillary type	Pedunculated	3
	Broad-based	3
Nodular type		5
Diffusely infiltrating type		13
Total		24

TABLE III

Histological Features of Carcinoma for Various Gross Forms

Gross forms	Histology	Cases
Papillary type	Ca. aden. papill.	6
Nodular type	Ca. solid. alv.	3
	Squamous cell carc.	1
	Ca. aden. simpl.	1
Diffusely infiltrating type	Ca. solid. diff.	4
	Ca. solid. alv.	2
	Ca. aden. papill.	3
	Ca. aden. simpl.	4
Total		24

In Table III are given the microscopic diagnoses of the main tumors of these cases, for each one of their gross forms. As to the characteristic features in their histology, refer to Figs. 6, 7, 8 and 9.

The rate of the lymph-nodal involvement, meatstasis to the liver or

TABLE IV

Spread of Lesion in Carcinoma of Gallbladder

	No. of cases with*		
	Lymph-nodal involvement	Metastasis to the liver	Infiltration to the serous membrane
Papillary type	2/6	2/6	0/6
Nodular type	5/5	3/5	2/5
Diffusely infiltrating type	12/13	4/13	13/13

* Denominators show the total numbers.

the invasion to the serous membrane in these cases is shown in Table IV, there being found a comparatively high occurrence of the incipient figures of malignant spreads, already at the time of operation.

Early carcinoma lesions found in the carcinomatous gallbladders

Two of the above-mentioned 24 cases showed the figures of early carcinoma originated in pre-existed papillomas, and eight had the accessory, early malignant lesions beside the main tumors, ranging from one to three in number. The location of the lesions in these ten cases with early carcinomas is outlined in Fig. 10.

In Case 1, two papillomas were found, being about the size of a finger tip and of a pigeon's egg, respectively. Microscopically, both of them presented the characteristic features of papilloma being composed of the network of the coralliform stroma and of a layer of the high-columnar epithelium covering it. And in the latter, a lesion of carcinoma in situ was discovered (Fig. 11).

Though Case 2 was an aggravated papilloma showing almost similar histology to Case 1, the carcinoma in situ in this case showed, in part, the infiltration (Fig. 12).

In the above two cases, besides, papillary proliferations of the epithelium were recognized even at the outside of the mentioned main lesions, which were thought to be incipient figures of papilloma. Especially in Case 2, such a proliferated epithelium showed, in part, an apparent evidence of atypicality and was considered to be a precancerous lesion. Recently, similar cases viz. papillomas of the gallbladder accompanied with carcinomatous aggravations were reported also by other authors, for example, a case by Nagai[2] and Miyaji,[3] respectively, and three cases by Tabah.[4]

In the other eight cases, Cases 3 to 10, one to three foci of early carcinoma were observed microscopically beside the main tumor (two cases of the broad-based papillary type, three of the nodular type and three of the infiltrating type), as stated above.

Gallbladder walls of Cases 3 to 9, the majority of these cases, presented the figures of severe chronic inflammation, these mucous membranes being rather atrophic in general. And in the latter, there were found scattered foci of papillary or adenomatous growths of the epithelium, a part of which showed the obvious atypicalities such as variations in size of the epithelial cells, polymorphies, enlargement of the nuclei or disappearance of the polarity.

In some instances, such papillary or adenomatous growths with the atypical structure showed marked irregularities, invading the stroma with the irregular nests or cords of the proliferated cells, initial figures of the infiltrating growth were thus observed (Fig. 13).

In Case 10, the mucous membrane of which was hypertrophic and a focus of carcinoma in situ was observed therein, infiltration into the stroma was not yet observed (Fig. 14).

In the above-mentioned eight cases, Cases 3 to 10, histological studies of the serial sections failed to detect the patho-histological continuity between the main tumor and any one of the accessory early malignant lesions. Moreover, the main tumor of Case 6 was squamous cell carcinoma, while the early carcinoma lesions of the same case were adenocarcinoma composed of the high-columnar cells.

These interesting findings suggest that the carcinomatous foci of the gallbladder mucosa in such cases had possibly been initiated " multicentrically ". Already Willis[5] noticed that the early carcinomas of the gallbladder were not unusually of the multicentric or the wide-spread and progressive origin.

III. Epithelial Overgrowths in Calculous or Inflammatory Gallbladder

It is a fact of a clinical evidence that the carcinoma of the gallbladder is often accompanied with calculi, the reported rate of the concurrence being as shown in Table V. As to our cases, 15 (62.5 per cent) of 24 carcinomas of the gallbladder were complicated with cholecystolithiasis. The number of the calculi in our cases was one to nine in eight cases and ten or more in the other cases, the largest one being 46.

Such a not unusual concurrence of the carcinoma and the calculi may suggest the pathogenetic relation between them. Therefore we performed thorough examinations of the 237 gallbladders ectomized for various cholecystic disorders other than neoplasms, especially intending to discover the papillary or adenomatous proliferations or the precancerous gorwths.

Relation with the calculi

Among the mentioned 237 cases, 168 were calculous and 69 were non-calculous. The results are summarized in Table VI.

TABLE V

Rate of Concurrence of Calculi in Carcinomatous Gallbladder

Authors	Total cases	Concurrent cases
Judd	56	53 (94.4%)
Russel	22	19 (86.4%)
Johnes	50	41 (82.0%)
Courvoisier	84	74 (76.2%)
Arminski	2068	1514 (73.2%)
Smithies	23	16 (69.6%)
Ourselves	24	15 (62.5%)

TABLE VI

Rate of Occurrence of Anomalously Proliferated Epithelium in Calculous and in Non-calculous Gallbladders

	Total cases	Papillary or adenomatous proliferation	Precancerous growth
Calculous	168	25 (14.6%)	7 (4.1%)
Non-calculous	69	6 (8.7%)	1 (1.4%)
Total	237	31 (13.1%)	8 (3.3%)

In 25 (14.6 per cent) of the 168 calculous cases anomalous proliferations of the epithelium, either papillary or adenomatous, were observed, seven (4.1 per cent) of which were revealed to be precancerous. Among the 69 cases with non-calculous disorders, on the other hand, such epithelial overgrowths were found in six cases (8.7 per cent), precancerous growth being observed in only one of them.

Relation with the inflammation

All of the mentioned 237 gallbladders were complicated with inflammations, whether acute or chronic and whether marked or mild. These were then studied for the relation with the anomalous proliferation of the epithelium.

Then, all of the 13 cases with marked acute inflammation showed no sign of the epithelial overgrowth, whereas many of the cases with chronic inflammations were accompanied with the evident anomalous proliferations of the epithelium (Table VII). Thus, among the 131 cases with mild chronic inflammations, whether with or without acute inflammation, six (4.6 per cent) showed the epithelial overgrowth, one (0.7 per cent) of which was thought to be a precancerous lesion; and among the 93 cases with marked chronic inflammation, also with or without acute

pictures, such overgrowths were found in 25 cases (26.9 per cent), seven (7.5 per cent) out of them being obviously precancerous.

From these results it was revealed that the anomalous proliferations of the epithelium of the gallbladder are most common in cases with marked chronic inflammation.

TABLE VII

Rate of Occurrence of Anomalously Proliferated Epithelium for Each Sort of Inflammations

	Total cases	Papillary or adenomatous proliferation	Precancerous growth
Acute inflammation	13	0 (0.0%)	0 (0.0%)
Chronic inflammation Mild	131	6 (4.6%)	1 (0.7%)
Chronic inflammation Marked	93	25 (26.9%)	7 (7.5%)
Total	237	31 (13.1%)	8 (3.3%)

Relation with the lapse of time since onset of the disease

Next, the rate of occurrence of the epithelial overgrowths was studied according to the lapse of time from the onset of the disease, which was presumed from the history of patient, to the operation.

As seen in Table VIII, the rate of the occurrence of the anomalous epithelial proliferation was 9.5 per cent in 147 cases which had been operated within five years from the onset of the disease, while that of

TABLE VIII

Relation between Epithelial Proliferation and Lapse of Time since Onset of Cholecystic Disorders

Lapse of time (years)	Total cases	Papillary or adenomatous proliferation	Precancerous growth
0—— 1	86	9 (10.5%)	1 (1.2%)
2—— 4	61	5 (8.2%)	2 (3.3%)
Total	147	14 (9.5%)	3 (2.0%)
5——11	59	13 (22.1%)	4 (6.8%)
11——44	31	4 (12.9%)	1 (3.2%)
Total	90	17 (18.9%)	5 (5.6%)
Total	237	31 (13.1%)	8 (3.3%)

the 90 cases operated five years or more after the onset was as high as 18.9 per cent.

To conclude, from the histological examinations of the 237 gall-

bladders which were ectomized for surgical disorders other than neoplasms, the anomalous proliferations of the epithelium were discovered in 31 (13.1 per cent) of them. Though such epithelial overgrowths were more common in calculous cases than in non-calculous ones, these were revealed to be correlated much more closely with the presence of the inflammations, being observed in highest rate in the cases with marked chronic cholecystitis.

Summary

1. Twenty four cases with carcinoma of the gallbladder were studied histologically. In ten gallbladders, one to three early microscopic lesions of carcinoma were observed beside the main tumors, suggesting that the multicentric occurrence of the lesion is not unusual.

2. Among the above 24 cases, 15 (62.5 per cent) were complicated with calculi. Such a high rate of the concurrence suggested some relations between the two conditions, therefore thorough microscopic studies were performed on 237 gallbladders which had been ectomized for various surgical disorders other than neoplasms.

3. The papillary or adenomatous proliferations of the epithelium were observed in 31 (13.1 per cent) of the 237 cases, i.e. in 25 (14.6 per cent) of the 168 calculous and in six (8.7 per cent) of the 69 non-calculous cases, among which eight cases were evidently precancerous.

These epithelial overgrowths were, however, more closely dependent upon the existence of the inflammations than the presence of calculi, and these were observed most commonly in cases with the marked chronic inflammations.

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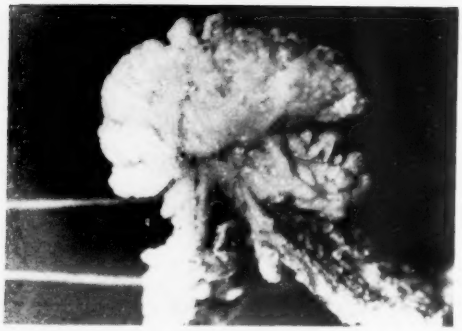

Fig. 1. Carcinoma of the gallbladder.
Pedunculated papillary type, which was revealed histologically to be an early carcinoma and to have occurred in a pre-existed papilloma.

Fig. 2. Carcinoma of the gallbladder.
Broad-based papillary type (shown by an arrow) originated in the neck of the gallbladder.

Fig. 3. Carcinoma of the gallbladder.
Two tumors of the nodular type.

Fig. 4

Fig. 5

Fig. 4. Carcinoma of the gallbladder.

Diffusely infiltrating type. In this case, the lesion (shown by arrows) is limited to only a portion of the gallbladder.

Fig. 5. Carcinoma of the gallbladder.

Diffusely infiltrating type. Almost all areas of the mucous membrane are involved. Notice the extreme constriction of the gallbladder. Outside of the bladder are shown the co-existed calculi.

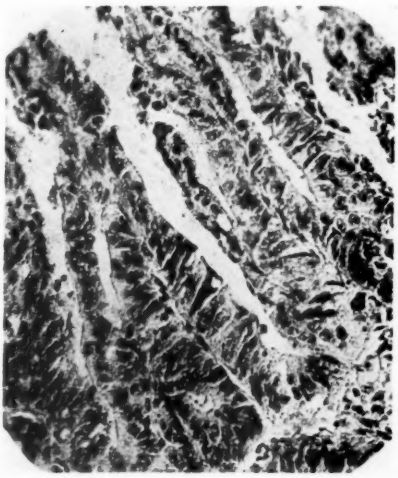

Fig. 6. Carcinoma adenomatosum papilliferum, constructed of high-columnar cells.

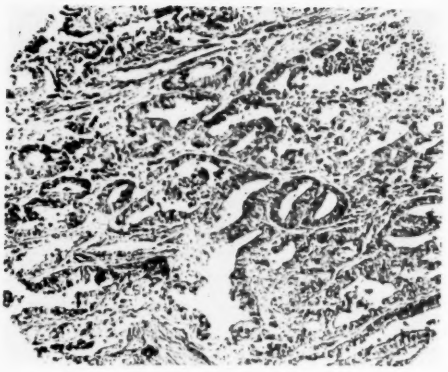

Fig. 7. Carcinoma adenomatosum simplex.

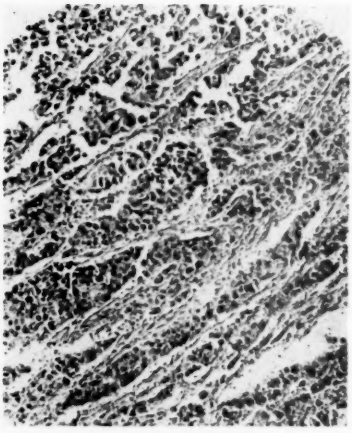

Fig. 8. Carcinoma solidum alveolare.

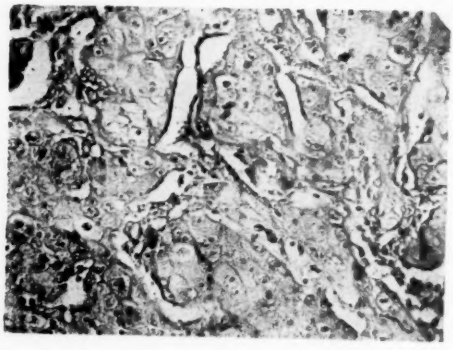

Fig. 9. Squamous cell carcinoma.

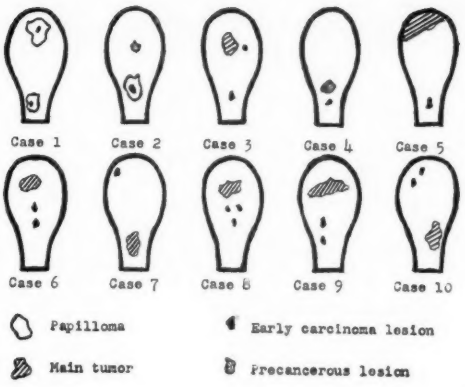

Fig. 10. Diagram showing the site of the early carcinoma or the precancerous lesions.

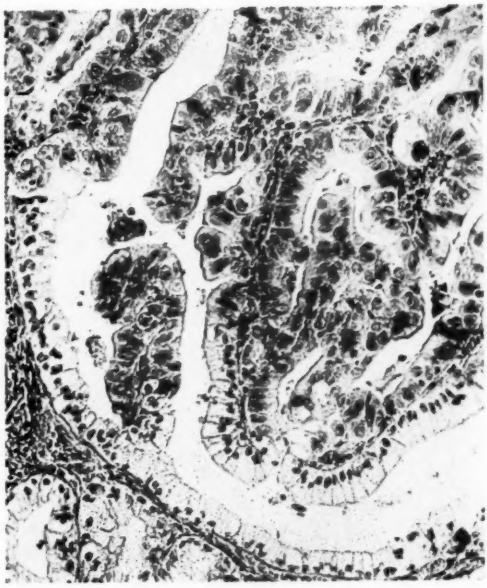

Fig. 11. Carcinoma in situ observed in a papilloma. In part, the cells are not yet malignant, showing the proper structure of papilloma.

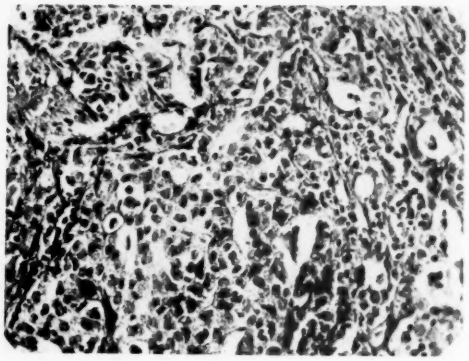

Fig. 12. Early features of the infiltrating growth observed in a part of the carcinoma in situ.

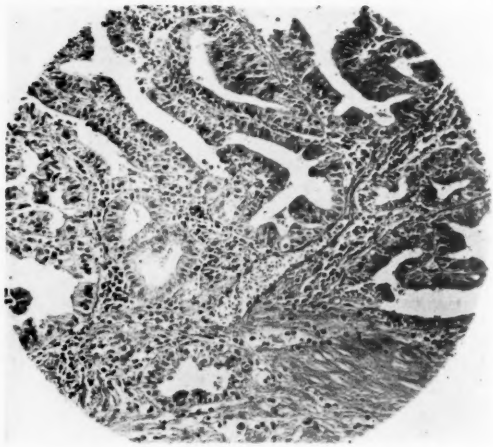

Fig. 13. Early carcinoma lesion observed in the papillarily proliferated epithelium. Incipient features of the infiltrating growth are seen, in part.

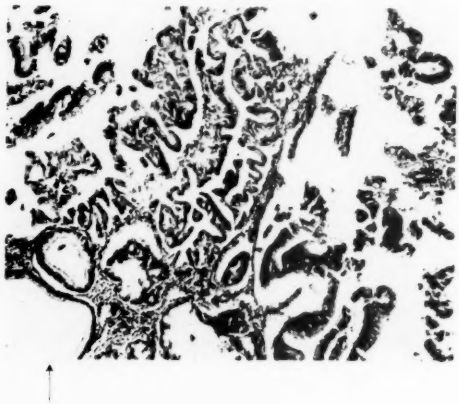

Fig. 14. Carcinoma in situ in Case 10.

A part of the cells (shown by an arrow) is high-columnar in form, remaining uninvolved.

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Studies on Experimental Silico-Tuberculosis Comparative Study with Dusts of Varying Contents of Free Silica

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Introduction

Rössle *et al.* in 1921 advocated a theme that the presence of silica in the silico-tuberculous lungs had benificial effects by producing productive changes on the tuberculous process. This conclusion was based on the data obtained from autopsy cases, incidence rate of tuberculosis patients in local districts where occupational silicosis was prone to prevail and the several results of their experiments on animals, and had been supported by several pathologists. Since then, however, a lot of informations have been accumulated and a number of objections have been raised against this opinion of Rössle *et al.* For an example, Akazaki *et al.*, studied 49 autopsy cases of miners with pulmonary silicosis from Sado and Osarizawa Mine, of Mitsubishi Metal Mining Co., of which 36 cases or 73.5% of the total had pulmonary tuberculosis as a complication. In this study they confirmed that the tuberculous changes as a complication revealed to be of the exudative type in the majority of these cases.

Later on, Ishii, one of our coworkers, infected rabbits with tubercle bacilli before or after let them inhale fine dust of quartz from the said Sado Mine and demonstrated that the tuberculous changes in the silicotic animals were much more aggravated than those in the control animals. On the other hand, we have reports on clinical and statistical observations indicating that the manifestations of silicosis found in different mine districts pulluted by dust containing free silica differed from each other. In this connection a hypothesis that the developmental difference of silico-tuberculosis depends on the quantity of free silica particles in the inhaled dust has been propounded (Sano) and yet it has not been experimentally tested. The present authors, aimed to verify the above assumption experimentally, and employed the same method as of Ishii using samples

of dusts from mines with high and low silica contents.

Experimental Materials and Methods

I. Materials

1. Dust Samples: For preparing dust with high content of free silica, we used quartz dust from Sado Mine of Mitsubishi Metal Mining Co., (containing 91.28% of free silica) and for dust with low content of free silica, gangue minerals from Hosokura Mine of Mitsubishi Metal Mining Co. (containing 38.60% of free silica) were used. These materials were ground into fine dust of ca. 1 μ in size.

2. Experimental Animals: The animals used in this investigation were matured rabbits of more than 6 months old.

3. Tubercle Bacilli: Vital low-virulence bovine type bacilli were used for infection; suspended in physiological saline so that 0.025 mg. of bacilli (the dosis found proper by preliminary tests) was contained in 0.5 cc. of saline.

II. Methods

Sixteen rabbits were divided into 4 groups (detailed below) and to each of them control groups of simple silicosis and simple tuberculosis were assigned.

1. Group Ls of animals inhaled dust with low silica content for a short period (about 300 inhalation times within 268–474 days) and then infected with tubercle bacilli.

2. Group Ll of animals inhaled dust with low silica content for a longer period (about 500 inhalation times within 484–896 days) and then infected with tubercle bacilli.

3. Group Hs of animals inhaled high-silica content dust for a short period (as in Group Ls above) and infected with tubercle bacilli.

4. Group Hl of animals inhaled high-silica content dust for a longer period (as in Group Ll above) and then infected with tubercle bacilli.

Once or twice a day, the animals were put into specially constructed inhalation boxes which were placed in a semihermetic room containing the silica dust. A round of dusting consisted of 5 minute blowing by a 1/3 hp. ventilation motor followed by one hour stay in the inhalation box so as, to have the rabbits inhale the dusty air spontaneously. The number of dust particles in the inhalation boxes was listed in Table I.

After the inhalation period, the rabbits were given 65 days' rest without further inhalation, and then a hematogenous infection was made to all the animals by introducing 0.025 ffig. of low-virulence bovine type tubercle bacilli through their auricular vein 50 days after this, they were sacrificed by air embolism and their lungs and some other organs were examined. The data are shown in Table II.

TABLE I

Number of Dust Particles in the Inhalation Box

	Number of dust particles per 1 cc of air
Immmediately after dusting	152.364
30 min. after dusting	15.381
60 min. after dusting	6.718

TABLE II

Specification of Experimental Conditions

Group	Rabbit No.	Rounds of inhalation	Days of inhalation	Rest before tubercle infection	Dosis of tubercle infection	Rest after tubercle infection	Remarks
		times	days	days	mg	days	
Ls	21	300	474	65	0.025	50	Sacrificed
	22	301	474	"	"	"	"
	25	312	384	"	"	"	"
	30	332	287	"	"	"	"
Ll	20	425	484	"	"	"	"
	53	493	896	"	"	"	"
	59	502	839	"	"	"	"
	54	514	896	"	"	"	"
Hs	31	297	268	"	"	"	"
	27	314	368	"	"	34	Died spontaneously
	28	324	287	"	"	50	Sacrificed
	26	324	384	"	"	"	"
Hl	60	517	710	"	"	"	"
	70	517	710	"	"	"	"
	52	522	894	"	"	"	"
	76	527	847	"	"	"	"
Simple silicosis controls to Ls	23	276	326				Died spontaneously
	29	333	231				Sacrificed
							"
Ditto to Hs	74	239	466				"
	51	297	559				"
Ditto to Hl	71	480	742				"
	73	504	820				"
Simple tuberculosis control	1		50		0.025		Sacrificed
	2		50		"		"

Experimental Results

I. Group Ls:

As summarized in Table III, the findings in each rabbit of this group

TABLE III

Grade of Pathological Changes (Ls)

Group	Rabbit No.	Silicotic changes						Tuberculous changes							
		Dust-carrying cells in alveoli		In follicles of bronchial wall		Alveolar wall and stroma interstitium		Productive				Exudative			
		Grouped	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis	Number	Size	Necrosis	Bacilli	Number	Size	Necrosis	Bacilli
Ls	21	##	±	+	−	−	−	6	+	+	−	4	+~##	−	−
	22	+	+	±	−	+	−	6	+	−	−	6	##~###	±	+
	25	+	+	±~+	−	+	−	7	+~##	±	−	5	##	±	+
	30	±	±	+~##	−	+	−	7	+~##	+	+	2	+~##	+	+
Controls: simple tuberculosis	1							1	+	−	−	3	±	±	±
	2							15	+	−~+	+	4	+~##	+	+
Ditto: simple silicosis	23	+~##	±	+	−	+	−								
	29	+~##	±	+	−	+	−								

Note: −: negative, ±: very rare or very small, +: few, mild or small, ##: moderately numerous or large, ###: numerous, advanced or large (In all the tables here under) ### or ####: very numerous

were rather similar to one another, as with the findings in the controls of the same group.

As shown in the table, the silicotic changes in the Group Ls and control group (subjected only to dust inhalation under the same conditions) were slight and similar whereas the tuberculous changes were obviously more aggravated in the Ls group than the control group subjected only to tuberculosis infection, revealing the lesions larger and tending to be exudative in the former.

II. In Group Ll:

The findings, similar in all the animals in the group, are given in Table IV, together with the findings in the simple tuberculosis controls. For the simple silicosis controls of this group, vide the note in Table I.

In summary, we may say that in all the 4 animals of Group Ll dust particles were phagocytized by dust cells and accumulated in the alveoli near the bronchioles. The alveolar walls showed some granulomatous swelling and granulomata were also formed in the peribronchial lymph follicles. Thus, in this group, the silicotic changes obviously milder than

in Group Hl subjected to inhalation of high-silica dust for nearly the same period and silicotic granulomata of the alveolar walls and the perivascular tissue were small with minimum collagenous fibres. The tuberculous changes, however, were found markedly aggravated, admitting that the degree of these changes vary individually. When we compared the findings of rabbit No. 53 with the mildest changes in the group and the control rabbit No. 2 with simple tuberculosis showing comparatively advanced tuberculous changes, the changes were obviously more advanced in the former than in the latter. In this group, the lesions were numerous and often of an exudative type, with occasional central necrosis. These necrotic foci were infested by some tubercle bacilli. These changes were more extensive with tendency to be an exudative form in this group than in Group Ls.

III. In Group Hs

The results are shown in Table V, in collation with those in the control groups concerned.

In spite of the similarity of the inhalation condition, the animals in this group showed considerable individual differences in their findings. For an example, while the rabbit No. 28 had only a slight thickening of the alveolar walls, the rabbits No. 26 and No. 27, had submiliar silicotic granulomata in the alveolar walls with some fibrous tissue proliferation. Also noted is a distorted structure of peribronchial lymphfollicles where the reticular cells that had taked up dust particles formed perceptible nodules with fibrosis. As yet the degree of difference of silicotic changes in this group and in the simple silicotic control group was slight and no evidence of aggravation of the silicotic changes secondary to the tuberculous changes noted. As to the tuberculous changes, the animals did not always show uniform changes, and in some cases, the findings were of a productiv type similar to those in the controls. In general, however, when the findings of the lungs of individual cases were summed up, the tuberculous lesions in this group were aggravated as seen by their number, size and infiltrative process into the surrounding tissue than those in the controls; the tendency to proceed to necrobiosis was also stronger in the former. As stated before, tubercle bacilli were found in a large number in the necrotic foci where it was rather infrequent in the control animals. The tuberculous lesions observed in this group were legs in number but larger in size, predominantly to be exudative and further advanced than those in Group Ls, but milder than those in Groups Ll in these respects.

IV. In Group Hl

The results are shown in Table VI.

In this group, the individual findings also varied as in the preceding. In the rabbits No. 52, 60 and 76, incipient but typical silicotic granulomata,

TABLE

Grade of Pathological

Group	Rabbit No.	Silicotic changes					
		Dust-carrying cells in alveoli		In follicles of bronchial wall		Alveolar wall and interstitium	
		Grouped	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis
Ll	20	++	+	+~++	−	+	−
	53	+~++	+	+	−	+	−
	59	+++	+~++	+~++	−~±	+~++	−
	54	+~++	+	±~+	−	+	−
Controls: simple	1						
Tuberculosis	2						

TABLE

Grade of Pathological

Group	Rabbit No.	Silicotic changes					
		Dust-carrying cells in alveoli		In follicles of bronchial wall		Alveolar wall and interstitium	
		Grouped	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis
Hs	31	++	++	+~++	+	+	±
	27	+	+	++	+	+	±
	28	+~++	+	+	+	+	±
	26	+	+	++~+++	+	+~++	±
Controls: simple	1						
Tuberculosis	2						
Ditto: simple	74	±	±	±	±	±	−
Silicosis	51	+	+	+	±	+	±

sometimes accompanying more or less perceptibele collagenous fibrosis, were found not only in the peribronchial lymph follicles but also in the alveolar wall. Despite more than 100 days elapsed after their last dust inhalation, often enough, aggregates of cells containing dust particles were found in foci of alveoli. The walls adjacent to such lesions were thickened and in some cases granulomata were beginning to be formed. The associated tuberculous changes were always far wider-spread than in the simple tuberculosis controls; in particular, in No. 76 they were consisted mainly of exudative foci with extensive central necrosis. In comparsion

IV
Changes (Ll)

Tuberculous changes							
Productive				Exudative			
Number	Size	Necrosis	Bacilli	Number	Size	Necrosis	Bacilli
24	+	±	−	14	+∼卄∼卅	卄	卄
9	+	±	−	3	+∼卄	±∼+	+
11	+	±	−	9	+∼卄	±	+
23	+	±∼卄	±∼+	5	+∼卄∼卅	±∼卄	卄
1	+	−	−	3	±	±	±
15	+	−∼+	+	4	+∼卄	+	+

V
Changes (Hs)

Tuberculous changes							
Productive				Exudative			
Number	Size	Necrosis	Bacilli	Number	Size	Necrosis	Bacilli
3	+	−	−	8	+∼卄	±∼+	卅
3	+	−	−	3	+	±	卄
2	+	±	−	3	+∼卄	+	卄
11	±∼+	±	−	7	+∼卄	±∼+	卄
1	+	−	−	3	±	±	±
15	+	−∼+	+	4	+∼卄	+	+

with the findings in Group Hs above, in this group the lesions were considerably much larger both in size and number, and the tuberculosis was much more aggravated. In some cases, the lesions were well-encapsulated, productive in type, but the aptness to become exudative and necrosis were generally stronger here than in Group Hs. The changes in this group were more advanced than those in Group Ll too, both in extent and aptness to become exudative. The severity of the tuberculous changes in Groups Ll and Hl might be related with the abundance of dust cells in the alveoli.

TABLE

Grade of Pathological

Group	Rabbit No.	Silicotic changes					
		Dust-carrying cells in alveoli		In follicles of broncaial wall		Alveolar wall and interstitium	
		Grouped	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis
Hi	60	###	##	###	+	##	+
	77	###	##	##	+	##	±
	52	+	±	##	##	+	+
	76	###	##	###	+	##	+
Controls: simple Tuberculosis	1						
	2						
Ditto: Simple silicosis	71	+	±	##	±	+	±
	73	##	+	###	+	+	+

Discussion

In reviewing the above experimental results, we find that the tuberculous changes in silicotic rabbits were always much more aggravated than in non-silicotic controls, regardless of difference of the free-silica content in the inhaled dust or the length of inhalation. The silicotuberculous changes tended to be more advanced in the groups subjected to longer exposure to dust, and in somewhat severer in the groups subjected to inhalation of dust with higher silica content, though the degree was much lower than the former group.

In the rabbits made to inhale dust with high-silica content, the accumulation of dust cells in the alveoli was larger than in the other rabbits, and among rabbits made to inhale dust of the same silica content, those subjected to more frequent rounds of inhalation had more dust-cells in their lungs.

It may be readily understood that in the lungs, having (anatomically) intricate vascular and air way systems with oxygen supply suitable for the growth of tubercle bacilli, the tubercle bacilli would be more apt to be detained there when hematogenous infection is effected after dust inhalation, than in normal lungs, and that the detained bacilli would aggravate the pathological changes, with their own virulence together with the organ disposition of the lung and further by the pernicious effect of the inhaled dust.

The fact that the tuberculous changes in the lungs of experimental animals are found aggravated even in the cases without fibrosis, seems to

VI
Changes (Hl)

Tuberculous changes							
Productive				Exudative			
Number	Size	Necrosis	Bacilli	Number	Size	Necrosis	Bacilli
7	+	−	−	18	++~+++	+	+
7	+~++	±	−	12	++~+++	+	+
7	+	±	−	5	++~+++	±~+	++
3	+	±	−	20	++~+++	++	++
1	+	−	−	3	±	±	±
15	+	−~+	+	4	+~++	+	+

suggest that the very existence of dust particles or dust-cells in the alveoli plays a major role in the tuberculous process. At least, we cannot neglect this fact in discussing the status of tuberculous changes associated with pneumoconiosis.

We might here cite the report of Kettle (1924) who made relevant experiments, in different sites and by a different method of ours. He found in white rats that following subcutaneous injection, the tubercle bacilli were largely proliferated in the sites where silicic acid was abundant, and expressed that silica dust may serve in fostering the growth of tubercle bacilli. Price (1932), Gardner (1934), Vorwald (1937) and Holzapfel (1941) concurred with him. In our experiments, we found that the longer the exposure of the animal to the dusty air the larger the accumulation of dust-cells in the alveoli. From such finding, it may be inferred that the quantity of silica dust or dust-cells accumulated in the alveoli influence the development of the tuberculous changes, perhaps due to the glutting of the phagocytes in the alveoli with silica dust particles, leading to drastic enfeeblement of their natural anti-tubercle-bacillus function.

Using rabbits with simple silicosis as controls, the reverse relation, if the silicotic lesions would be affected by tuberculous infection, was also examined, but no evidence of increase or enlargement of silicotic granulomata following tuberculous infection was observed.

Summary

1. The tuberculous changes induced in the experimental silicotic

lungs of rabbits were found perceptibly more aggravated than those in the control rabbits with simple tuberculosis.

2. In groups of animals subjected to nearly the same rounds of dust inhalation, the tuberculous lesions were somewhat more aggravated in the animals subjected to inhalation of dust with high silica content than those given low silica content, but the difference was rather slight.

3. In the groups made to inhale dust with the same silica content, the animals subjected to more rounds of inhalation showed higher aggravation of the tuberculous lesions, in number, size and tendency to exudative changes, than those subjected to lesser rounds of dust inhalation.

4. The higher accumulation of silica-dust carrying cells in the cases with higher aggravation of the tuberculous changes seems to suggest that in accounting for the development of silico-tuberculous lesions we must pay more attention to the total amount of silica, especially, the dysfunction of the phagocytes due to their satiation with silica particles, rather than to the content of free silica in the dust.

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Fig. 1

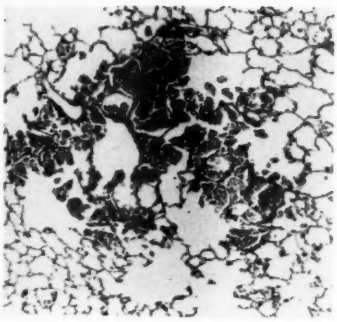

Fig. 2

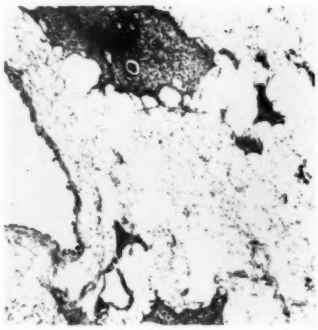

Fig. 1. Rabbit 23. (Control with simple silicosis. Ls).
Dust-carrying cells accumulated in alveoli and slight swelling of the adjacent alveolar walls but scarcely any fibrosis observable.
Fig. 2. Rabbit No. 60. (Hl. Tuberculosis infected. ×60).
Typical perivascular silicotic granulation tissue in formation.

Fig. 3

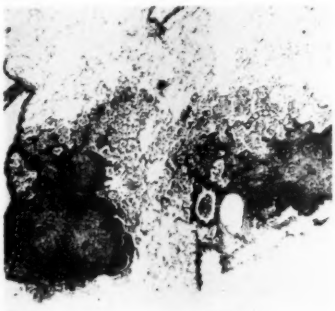

Fig. 4

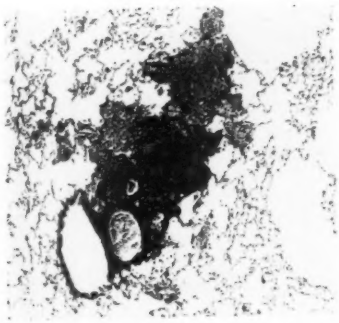

Fig. 3. Rabbit No. 2. (Control with simple tuberculosis. ×35)
Comparatively localized tuberculous nodule with slightly caseated center.
Fig. 4. Rabbit No. 2. (Ditto. ×60).
A tuberculous focus without marked exudative tendency.

Fig. 5

Fig. 6

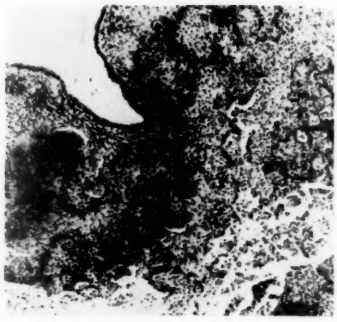

Fig. 5. Rabbit No. 30. (Ls. Tuberculosis infected. ×60).
Tuberculous focus larger than in No. 2 above.
Fig. 6. Rabbit No. 20. (Ll. Tuberculous. ×60).
Exudation in marked progress and the tuberculous focus markedly enlarged over that in No. 30 above.

Fig. 7

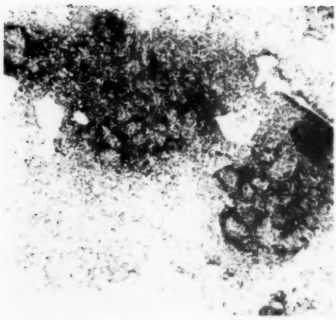

Fig. 8

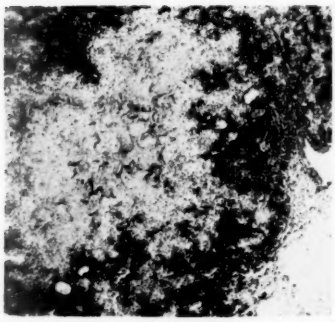

Fig. 7. Rabbit No. 31. (Hs. Tuberculous. ×35).
The tuberculous focus shows a marked exudative tendency as compared with that in No. 30 above but somewhat milder than that in No. 20 above.
Fig. 8. Rabbit No. 76. (Hl. Tuberculous. ×35).
The exudative tuberculous focus the largest in all the animals.

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On the Experimental Anthracosis, Anthracosilicosis and the Relationship of these to Tuberculosis in Complication

By

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Introduction

Throughout the last century, in Europe the opinion was prevalent that ' anthracosis is not subject to complication with tuberculosis or if such a complication ever occurs in, the disease progresses favorably,' but more recently, severe criticisms have been raised against this optimistic opinion. For an example, Shirakawa, basing himself on the results of wide-scoped statistical and clinical studies covering workers in Hokkaido Colliery (Japan) and experimental researches, asserted that there was no specificity in the problem of tuberculosis among coal miners, and that ever a higher incidence of tuberculosis was observed among the workers subject to larger inhalation of coal dust. Lately, Cochrane, upon statistical observations, concluded that the inhalation of small quantity of coal dust acted in lowering the tendency of aggravation of tuberculosis, but its heavy inhalation caused uprise of the rate of tuberculous complication of anthracosis and the mortality.

Experimental studies for elucidating the correlation between anthracosis and tuberculosis associated with it are not rare, but analytical studies with adequate attention to the precise chemical composition of the inhaled dust seem to be regrettably scarce.

Akazaki, one of the present authors, in his study on the correlation between silicosis and tuberculosis complicated therewith, in collaboration with Matsuoka, has demonstrated that the large accumulation of dust-cells that have taken the inhaled silica dust in the lung by itself effects aggravation of the tuberculous complication. Enterpreting this effect, the authors assumed this is resulted from the blocking of function of the dust-carrying cells that should have otherwise acted against the infecting tubercle bacilli. Such an assumption would naturally lead to the corollary that a heavy inhalation of dust that is powerless by itself in inducing fibrosis in

the lung will eventually aggravate tuberculosis in complication.

The primary aim of the present study is, of course, to shed light on the relationship between anthracosis and the complicating tuberculosis, but further aim consists in the endorsement of the above interpretation of ours concerning silico-tuberculosis, through experiments with coal dust which unlike to silica dust, hitherto accepted as harmless to pulmonary tissue.

Experimental Materials and Methods

A. Materials

1. Animals and Bacilli: Rabbits of 2–3 kg. and in the course of maturing were used as experimental animals. For experimental tuberculosis infection, a specified strain of lowvirulence bovine type tubercle bacilli was used throughout.

2. Dust: Washed dust of coal and fine powder of gangue sandstone (particles below 1 μ: 30–50%, between 1 and 5 μ 45–55%) from Sakito Mine, Kyushu, where cases of pneumoconiosis and pneumoconiotuberculosis were detected in a rather large number at the mass-examination in 1954, were used.

Their chemical composition was as follows:

a) Composition of Gangue Mineral (%)

SiO_2	Fe_2O_3	Al_2O_3	MgO	CaO	MnO	Na_2O	SO_3	
67.06 including ca. 45 of free SiO_2	2.22	4.87	2.53	9.34	0.08	0.17	0.43	Remainder Zn, Cu, Pb, Sn

b) Composition of Coal (%)

Carbon	Hydrogen	Oxygen	Nitrogen	Sulfur	Ash
70.69	5.06	5.37	1.10	1.94	16.06

Composition of Ash (%)

SiO_2	Fe_2O_3	Al_2O_3	MgO	CaO	SO_3
51.47 including ca. 20 of free SiO_2	5.50	21.36	1.34	3.47	2.06

Thus, the total content of silica in the coal dust amounted to ca. 8.2%.

B. Methods

1) Dust Inhalation: Employing a specially devised dust inhaler, dust was blown into the dust boxes for 5 to 10 minutes a day, then the

animals were left in the dust boxes for 60 to 90 minutes. The concentration of dust in the air in the dust box was shown in the following Table:

Concentration of Dust in the Air in Inhalation Box

5 min. after start of dusting	ca. 152,000/cc
30 min. after dusting	ca. 15,300/cc
60 min. after dusting	ca. 6,700/cc

2) Tubercle Infection: The exact dosis of 0.025 mg. each was weighed out from the same culture of the above mentioned tubercle bacilli and introduced into the auricular vein of the rabbits to be infected. The introduction was carried out 59 days after the last dust inhalation and 50 days before their sacrifice.

3) Group of Animals: The simple pneumoconiotic groups consisted of the group of animals subjected to inhalation of coal dust alone (Group C) and another group of animals given coal dust and gangue dust mixed in equal parts, containing about 40% of total silica including about 25% of free silica (Group CS). The other hand as infective pneumoconiosis, several animals, each pre-treated similarly, were infected with tubercle bacilli as above (Groups CT and CST). The last group, of two rabbits infected with tubercle bacilli alone without dust inhalation, was kept as control. Experimental conditions and change of body weight of animals were shown in Table I–V.

Experimental Results

A. Histological Findings of Simple Pneumoconiosis Groups

Table I

Experimental Conditions and Change of Body Weight in Group C: of Animals Inhaled Coal Dust Alone

Group	Rabbit No.	Experimental conditions			Body weight		Remarks
		Rounds of inhalation	Days of experiment	Rest days after inhalation	Before inhalation	At death	
C	2	178	402	7	2500	1800	Died spontaneously
	6	180	391	1	2250	1700	"
	5	195	436	27	2500	1700	"
	9	300	554	1	2100	2500	Sacrificed
	13	312	468	109	2050	2200	"
	11	326	485	109	3000	2900	"
	8	492	894	109	2600	3800	"
	4	523	894	109	2100	3300	

TABLE II

Experimental Conditions and Changes of Body Weight in Group CS: of Animals Inhaled 50–50% Mixture of Coal Dust and Gangue Dust

Group	Rabbit No.	Experimental conditions			Body weight		Remarks
		Rounds of inhalation	Days of experiment	Rest days after inhalation	Before inhalation	At death	
CS	18	82	475	1	2700	2300	Died spontaneously
	19	175	370	84	2250	2100	"
	24	204	415	1	2100	2200	"
	26	325	438	109	2200	2200	Sacrificed
	17	436	736	2	2580	2400	"
	20	519	893	109	2400	3100	"
	22	520	814	30	2080	2200	Died spontaneously
	16	522	893	109	2630	3000	Sacrificed

TABLE III

Ditto in Group CT: of Animals Given Tubercle Bacilli After Inhalation of Coal Dust

Group	Rabbit No.	Experimental conditions			Body weight		Remarks
		Rounds of inhalation	Days of experiment	Rest days after inhalation	Before inhalation	At death	
CT	14	229	339	109	1900	3400	Sacrificed
	12	325	484	109	2600	2700	"
	3	354	753	109	2900	2600	"
	10	519	888	109	2000	3000	"
	1	523	894	109	2600	2600	"

1. Group C

The histological findings of the lungs of the animals in this groups were summarized in the Table VI:

In summary, we may say that in all cases, dust-carrying cells that had taken large quantities of dust were more or less accumulated in the alveoli (often filling up the alveoli in the cases subjected to dust inhalation for many days). In such alveoli filled with dust-carrying cells, the walls were granulomatously swollen with some interstitial dust particles and many phagocytes. Such findings were more frequently seen in terminal bronchioli and perivascular areas. Anthracotic granulomas were also

TABLE IV

Ditto in Group CST: of Animals Given Tubercle Bacilli After Inhalation of Coal Dust and Gangue Dust (50–50% mixture)

Group	Rabbit No.	Experimental conditions			Body weight		Remarks
		Rounds of inhalation	Days of experiment	Rest days after inhalation	Before inhalation	At death	
CST	28	74	157	109	2200	3000	Sacrificed
	27	307	428	109	2200	2600	"
	25	340	469	109	2100	2400	"
	23	519	888	109	2000	2400	"
	21	522	893	109	2200	2700	"

TABLE V

Group as Control: Two Animals Given Tubercle Bacilli Alone

Group	Rabbit No.	Days of experiment	Body weight		Remarks
			Before inhalation	At death	
Control	Tb. 1	50	2300	2250	Sacrificed
	Tb. 2	50	2800	2900	"

found in the stromal lymph follicles. In these lesions, however, only a very slight proliferation of argyrophil fibres could be observed, and proliferation of collagenous fibres was not perceptible anywhere. Thus, it was demonstrated that inhalation of coal dust alone did not induce formation of fibrous nodules.

2. Group CS

The histological findings of the lungs were summarized in Table VII.

In summary, we found that similar findings observed in the animals of Group C were also present in all the animals of this group; dust taken up by the reticulum cells or dust-carrying cells being accumulated in varying quantities in the alveoli, interstitium and peribronchial lymph follicles. In such lesions, the alveolar walls were diffusely swollen with increased cellularity, and irregular pneumoconiotic nodules were often formed surrounding the alveolar walls. Here, the cells were more abundant than as might be expected from the amount of dust taken up and sometimes even the picture of incipient hyperplasia of collagenous fibres without argyrophil fibres was also apparent. But such fibrosis cannot be deemed specific to silicosis and should be looked upon as representing

TABLE VI

Histological Findings in Group C (given coal dust alone)

Group	Case No	Pneumoconiotic changes							
		Dust-carrying cells in alveoli		In follicles of bronchial walls			In alveolar wall and interstitium		
					Fibrosis			Fibrosis	
		Agglomerated	Sporadic	Cell proliferation	Of argyrophil fibres	Of collagenous fibres	Cell proliferation	Of argyrophil fibres	Of collagenous fibres
C	2	±	±	±	−	−	−	−	−
	6	++	++	+	−	−	+	−	−
	5	+	++	+	−	−	±	−	−
	9	±	+	+~++	−	−	±	−	−
	13	+++	++	+	−	−	++	+	−
	11	+	++	+	+	−	±	±	−
	8	++	+	+	−	−	+	±	−
	4	+++	++	+	+	−	++	+	±

simple pneumoconiotic nodules.

B) Histological Findings of the Control Group with Simple Tuberculosis

See the following Table VIII.

In the two animals, despite same dosis of tubercle bacilli was administered, the tuberculous lesions showed considerable variety. The productive lesions were of milletsize and had epitheloid-cell infiltration at their center, with a surrounding zone, of lymphocytic infiltration and adjacent healthy lung tissue. Elsewhere, some exudative lesions were also present, where the proliferated histiocytes were often necrotized and, the surrounding lymphocytic zone was replaced by a large-size cell infiltration. In any case, no proliferation of collagenous fibres could be observed.

C) Histological Findings in the Pneumoconio-Tuberculous Groups

1. Group CT

In short, we might say that the pulmonary findings due to depositis of coal dust differ little from those in Group C above, having a picture of mere pneumoconiosis without fibrosis. However, it must be noted that the pulmonary changes in complication here were much severer than those in the animals of the simple tuberculous controls above. The lesions were much larger, with frequent exudative changes and marked necrosis. It

TABLE VII

Histological Findings in Group CS (given coal dust and gangue dust)

Group	Case No.	Pneumoconiotic changes							
		Dust-carrying cell in alveoli		In follicles of bronchial walls			In alveolar wall and interstitium		
					Fibrosis			Fibrosis	
		Agglomerated	Sporadic	Cell proliferation	Of argyrophil fibres	Of collagenous fibres	Cell proliferation	Of argyrophil fibres	Of collagenous fibres
CS	18	±	±	±	−	−	±	−	−
	19	±	±	±	−	−	±	−	−
	24	±	±	−	−	−	−	−	−
	26	+	++	+～+++	++	−	+	−	−
	17	+++	+	+++		−	+～++		−
	20	++～+++	+	++		+	++		±
	22	+	+	+	++	++	±	+	+
	16	+++	+	++	++	+	+++	++	+

TABLE VIII

Histological Findings in Simple Tuberculosis Controls

Group	Case No.	Tuberculous foci						Bacilli
		Productive			Exudative			
		Number	Size	Necrosis	Number	Size	Necrosis	
Control	Tb. 1	1	+	−	3	−	−	−～±
	Tb. 2	10	+	±	5	±～++	±	±～+

was also noted that the lesions were infiltrated with round cells, plasmocytoid cells, pseudo-eosinophilic cells and some others and showed rather complicated appearance, while anthracotic and tuberculous changes were mingled in many places. Such findings suggested that the anthracotic lesions induced first by the inhalation were invaded by the tubercle bacilli introduced later on which formed their own colonies there. It would indicate not that the pneumoconiotic lesions favored for the growth of

TABLE

Histological Findings in Group CT.

Group	Case No.	Pneumoconiotic changes					
		Dust-carrying cells in alveoli		In Follicles of bronchial walls		In alveolar wall and interstitium	
		Agglomerated	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis
CT	14	+	±	+	−	±	−
	12	+	±	+	−	++	−
	3	++	+	±	−	+	−
	10	+++	+	++	−	±	−
	1	+++	+	++～+++	−	++	−

TABLE

Histological Findings in Group CST. (given

Group	Case No.	Pneumoconiotic changes					
		Dust-carrying cells in alveoli		In follicles of bronchial walls		In alveolar wall and interstitium	
		Agglomerated	Sporadic	Cell proliferation	Fibrosis	Cell proliferation	Fibrosis
CT	28	±	±	±	−	−	−
	27	++	++	+	±	+	−
	25	++	+	+	+	+++	+
	23	+	+	+	+	+～++	+
	21	+++	++	++	+	+++	+

tuberculous bacilli but their locations at the terminal bronchioli suited for both the pneumoconiotic and the tuberculous lesions to be formed. Thus, the tuberculous lesions might frequently propagate with the pneumoconiotic lesions as starting bases. In any way, no fibrosis was observable in these cases of pneumoconiotuberculosis at all.

2) Group CST

The histological findings of the lungs were summarized in the following Table X.

It was apparent that the tuberculous changes were much more aggravated than in the control simple tuberculous group. In Case No. 23, the tendency to form exudation was stronger than in the animals of Group CT above and general tendency to become necrosis was also marked. In Case No. 27 the proliferative change was perceptible, together with the strong tendency to fall in necrosis. In particular it was also noticeable

IX

(given coal dust and tubercle bacilli)

Tuberculous foci						
Productive			Exudative			Bacilli
Number	Size	Necrosis	Number	Size	Necrosis	
3	+	−	12	+～卌	±	±
9	+	±	15	卄～卌	+～卄	+
10	+	±	30	+～卌	+～卌	卄～卌
3	+	±	10	卄～卌	+	+
6	+	−	14	+～卌	±～卄	卄

X

coal dust, gangue dust and tubercle bacilli)

Tuberculous foci						
Productive			Exudative			Bacilli
Number	Size	Necrosis	Number	Size	Necrosis	
6	+	±	8	卄～卌	+	+
12	+	+～卄	10	+～卌	+	+～卄
6	+	+	6	+	±	±
3	+	+	12	+～卌	+	+
8	+	±	20	+～卌	+	卄

that the degree of the silicotic changes, were in accordance with the tuberculous changes. Thus, even in Case No. 25 which was subjected to comparatively few rounds of dust inhalation, a rather prominent proliferation of collagenous fibres associated with, frank formation of pneumoconiotic granuloma or nodules were observed. This seemed to suggest an existence of the collaborative action between the tubercle bacilli and the silica dust particles.

Discussion and Summary

As already stated in the introductory chapter, it was our object to study experimentally how the tuberculous changes in the lung were affected by the pre-existing anthracotic changes. As stated above the mere inhalation of coal dust containing only 8.2% of silica (including 3% of free silica), despite repeated 523 times over a period of 894 days, elicited granu-

lomas of dust-carrying cells without fibrocollagenous proliferation. But using the coal dust mixed with powdered gangue mineral in equal parts so as to raise the total silica content to 40% and the free silica content to 25%, we succeeded in inducing obvious proliferation of collagenous fibres and formation of the so-called anthracosilicotic granulomata with conspicuously proliferated dustcarrying histiocytes, although the inhalation was not exceeded to the preceding case.

Rabbits with similary induced pneumoconiosis were then infected with very low-virulent tubercle bacilli. In the rabbits previously subjected to inhalation of pure coal dust, the super-imposed tuberculous lesion were found more aggravated than in the simple tuberculous controls, regardless of the duration of inhalation. The aggravation, though not extensive like that of the rabbits made to inhale silica dust which previously reported by Akazaki and Matsuoka, was still marked enough. Here, however, we could not detect any indication of the pre-existing anthracotic changes being aggravated by the later-coming tuberculosis.

In the rabbits previously inhaled coal dust mixed with equal quantity of silica dust and later on infected with tuberculosis, the tuberculous lesions were found frankly aggravated beyond those in the control animals, as in the preceding. Here however it was notable that the severer the pneumoconiotic changes, the worse were the tuberculous changes in many cases. Besides, the pneumoconiotic changes were also further advanced. These findings were somewhat different from the tuberculous changes in the lungs of rabbits made to inhale unadulterated coal dust.

Zeidi *et al.* assert upon their recent experiments that dust of coal mines and tubercle bacilli mutually act in aggravating the lesions. There is no objection against their opinion in advocating that the tuberculous changes aggravated by the presence of anthracosis, but with the other half concerning the aggravation of anthracotic changes by tuberculosis, we cannot concur off-hand. In the process of pneumoconiosis of coal miners aggravated by subsequent tuberculous infection, we should rather pay more attention to the presence of silica in the pit dust.

Finaly, we will refer briefly on the question how inhalation of dust causes aggravation of tuberculosis superinduced later on. The aggravation of tuberculosis observed in the present study, obviously, is not specific to silicosis. According to the experimental study by Yamamoto of this laboratory, in the rabbits made to inhale dust of sericite entirely harmless in causing fibrosis, the superinduced tuberculous changes were more aggravated in the animals subjected short period of inhalation than those of longer duration. From such findings we might deduce that inhalation of any dust would always aggravate the tuberculosis induced thereafter, at least experimentally, and it means merely silica dust effects most in

this respect.

Then, why does the presence of dust aggravate the tuberculous changes in the lung? It is clearly inferable from the results of the experiments by Yamamoto that the effect is not due to the obstruction of circulation of blood and lymph by the fibrosis and other changes caused by the dust. We must in this connection look for a factor common to silica dust and other dust effective in causing fibrosis as well as to sericite dust and coal dust devoid of such effect. From such a point of view, we must pay attention to the phenomenon of dust particles satiating the dust-disposing cells and thus blocking their function. Such dust-disposing cells should play key roles not only in the process of disposing dust in the lung but also in the fight against invading tubercle bacilli. The fact that the same terminal bronchioli offer preferential sites for formation of both the pneumoconiotic and the tuberculous lesions, as already stated repeatedly in the chapter on experimental results above, seems to contribute in expediting the aggravation of the tuberculous changes in complication.

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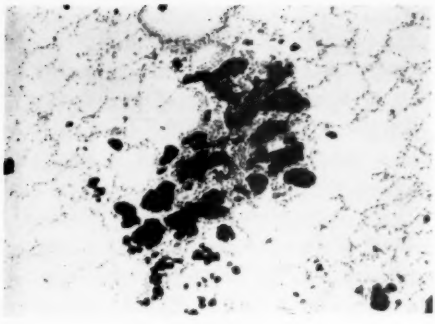

Fig. 1. Rabbit No. 13. (C. 312 inhalations).

Anthracotic focus in the right upper lobe. Dust-carrying cells aggregated in adjoining alveoli, the alveolar walls being thickened and showing slight cell proliferation. (H.E. staining ×100).

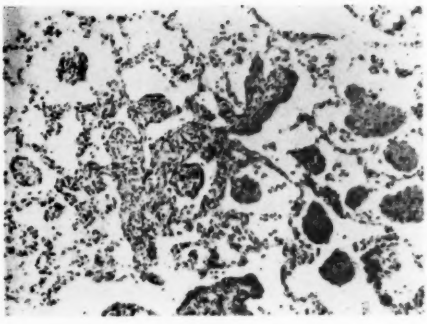

Fig. 2. Rabbit No. 26. (C+G. 325 inhalations).

Anthraco-silicotic focus in the right upper lobe. Obvious pneumoconiotic granulation nodule in formation in the alveolar wall. (H.E. staining. ×100).

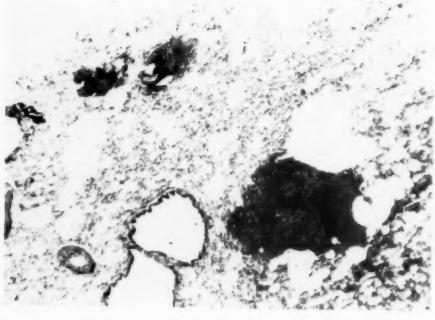

Fig. 3. Rabbit Tb. No. 2. (with simple tuberculosis. Control to No. 1–Fig. 5 and No. 23–Fig. 6).

Productive tuberculous focus in right upper lobe. The focus is relatively small and definitely marked off from the surrounding lung tissue. (H.E. staining ×35).

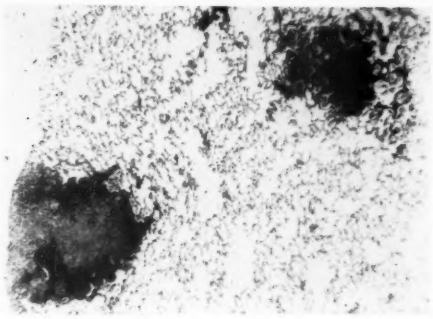

Fig. 4. Rabbit Tb. No. 2. (Ditto).
Productive tuberculous focus in left lower lobe. The focus is nearly equal in size to that in Fig. 5. above. Caseation at center and some exudative tendency observable. (H.E. staining ×35).

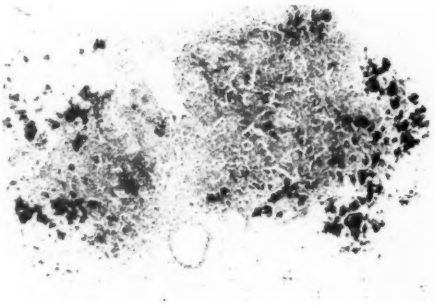

Fig. 5. Rabbit No. 1. (C. 523 inhalations and afterwards tuberculosis infected).
Tuberculous focus in left lower lobe. Accompanying marked accumulation of coal dust around it. (H.E. staining. ×35).

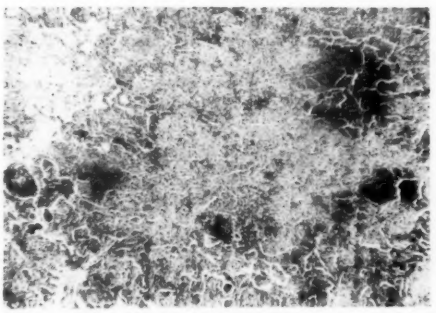
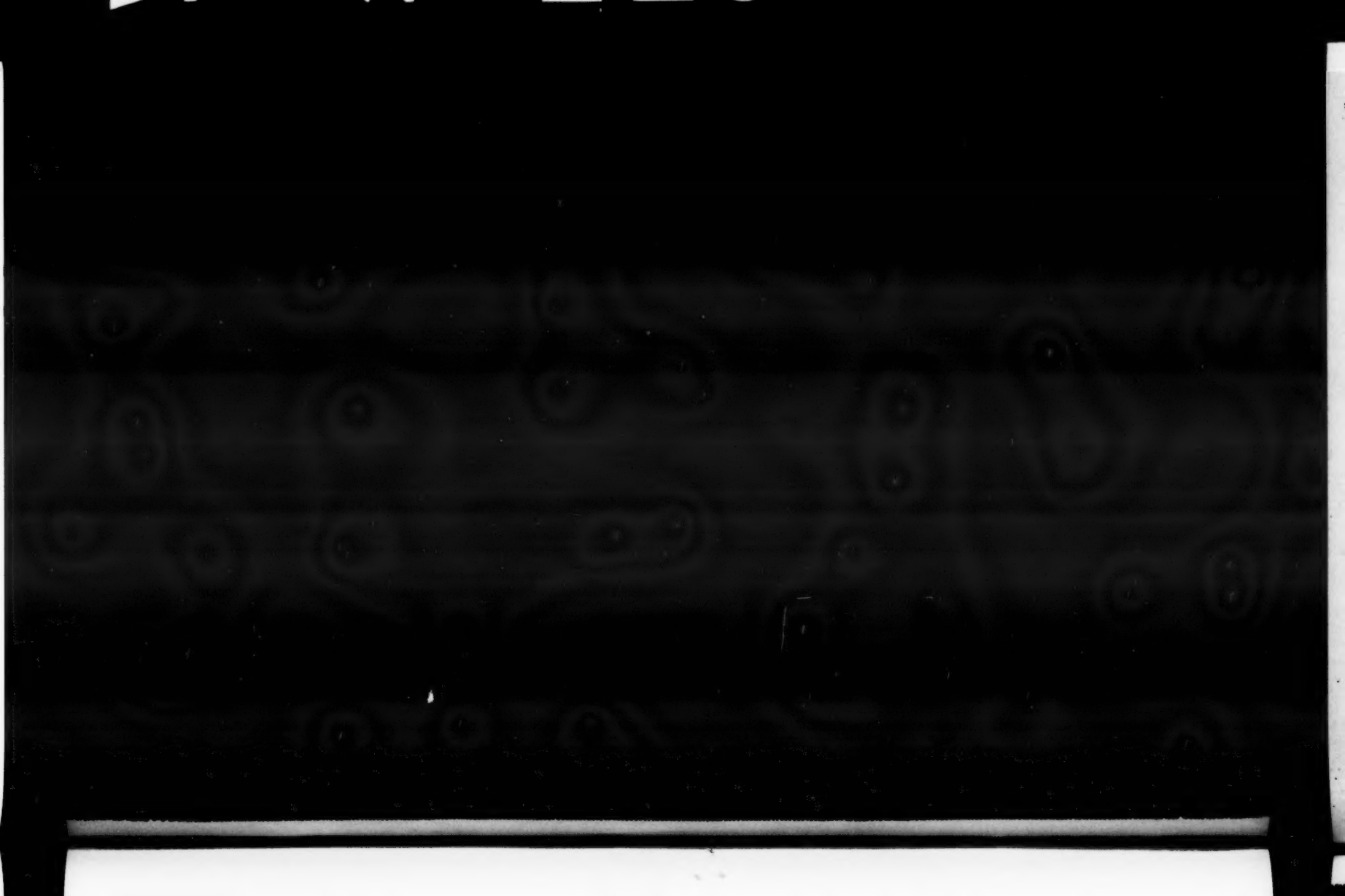
Fig. 6. Rabbit No. 23. (C+G. 519 inhalations and afterwards tuberculosis infected).
Tuberculous focus in right lower lobe. Highly exudative and necrosis frequent. (H.E. staining. ×35).